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Switching off the Fibroproliferative Phase of Wound Healing – An Investigation of the Normal Mechanisms and Pathological Scar-related Defects

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Dedication

I wish to dedicate this thesis to my family. To my parents, for their continued support, love and admiration for my work. To my sister for always listening and encouraging me. Finally, to my husband for being understanding, filling me with self-confidence and supporting me both financially and emotionally.

Thanks.

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Vigor C, Linge C, Sanders R. Aetiology of keloid scarring. Oral presentation, European Conference for Scientists and Plastic Surgeons. Geneva, Sept 2003.

Papers

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Abstract

Using *in vitro* models that mimic various aspects/stages of wound healing, this thesis attempted to define the events that lead to the culmination of the fibro-proliferative phase of wound healing, i.e. apoptosis and any potential defects exhibited by keloid scars. Normal scar-derived fibroblasts were found to undergo apoptosis in contractile collagen gels, whereas keloid fibroblasts did not.

Investigation of the mechanisms involved indicated that this form of apoptosis required both the three-dimensional and collagenous nature of the gel and was not simply caused by removal of tension, but required biochemical cues. Collagen-contraction-induced apoptosis was found to require matrix metalloproteinase (MMP) and autocrine transforming growth factor-β (TGF-β) activity. Indeed contraction was accompanied by significantly increased expression and activation of MMPs along with indications of increased matrix breakdown. Furthermore, pure products of matrix breakdown significantly induced apoptosis of normal scar cell monolayers.

The defect exhibited by keloid fibroblasts was found to be specific to that induced during collagen contraction since they were equivalent to normal scar cells in their sensitivity to other forms of apoptosis induction and demonstrated normotrophic p53 stabilisation and activation of PARP (Poly(ADP-ribose)polymerase) and caspase-3. During collagen contraction, keloid fibroblasts failed to produce biochemical cues of apoptosis and although they exhibited normal levels of MMP gene expression and activation, they failed to breakdown collagen gels. However, these cells did undergo apoptosis in response to the biochemical cues produced on normal scar cell contraction of collagen gels, but surprisingly could not respond to pure forms of matrix breakdown products. Unlike normal scar cells, keloid fibroblasts failed to differentiate into myofibroblasts in collagen gels. The addition of exogenous TGF-β1 was found to restore differentiation and furthermore allowed the cells to undergo apoptosis in collagen gels. Surprisingly, TGF-β1 also restored the ability of keloid cells to undergo apoptosis in response to matrix breakdown products.

Declaration of Originality

I declare that the laboratory research for this thesis is original and that the ideas were developed in conjunction with my supervisor. I would like to thank Janette Richardson for performing the experiments for the results presented on page 108 and 255. All other experiments were performed by myself with the guidance and technical assistance of the laboratory and scientific staff at the RAFT Institute of Plastic and Reconstructive Surgery, Mount Vernon Hospital.

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Abbreviations

A	Anchored
A+	Anchored + TGF-β
ADAM	A disintegrin metalloproteinase
ADAMT	A disintegrin metalloproteinase with a thrombospondin motif
ANOVA	Analysis of variance
Anti-TGF-β	Anti-Transforming growth factor-1 antibody
APAF-1	Apoptosis protease inducing factor-1
APS	Ammonium persulfate
ARTS	Apoptosis-related protein in TGF-beta signalling pathway
α-SMA	Alpha smooth muscle actin
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C	Contractile
C+	Contractile + TGF-β
C3	Complement factor-3
C4	Complement factor-4
CAD	Caspase-activated DNase
CARD	Caspase-associated recruitment domain
Casp	Caspase
Casp-3I	Caspase -3 inhibitor
CDK	Cyclin-dependant kinase
c-FLIP	Cellular FLICE-like inhibitory protein
$CHCl_3$	Isoamylalcohol
CTGF	Connective tissue growth factor
DAPI	4',6-Diamidino-2-phenylindole
DD	Death domain
DDM	Aspartate-aspartate-methionine amino acid motif
DED	Death effector domain
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DIABLO	Direct IAP binding protein
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleoside triphosphates
DPX	Dibutyl phthalate xylene
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
ED-A Fn	EIIIA fibronectin
EDTA	Ethylenediaminetetacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signalling-regulated-kinase
FAC	Focal adhesion complex
FADD	Fas adaptor death domain

FAK	Focal adhesion kinase
FasL	FAS ligand
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GT	Guanidine thiocyanate
GTPases	Guanadine thiosulphate phosphatases
HA	Hyaluronic acid
HB-EGF	Heparin-binding epidermal growth factor
HLA-C2	Human leukocyte antigen-class 2
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
ICAM-1	Intercellular adhesion molecule-1
ICE	Interleukin 1β-converting enzyme
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
IP-10	Inositol phosphatase-10
JNK	c-Jun-N-terminal kinase
KCL	Potassium chloride
KGF	Keratinocyte growth factor
KLD	Keloid
LAP	Latency-associated peptide
LPA	Lysophosphatidic acid
LTBP	Latent TGF-β-binding protein
M6P	Mannose-6-phosphate
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1alpha
MMP	Matrix metalloproteinase
MMP-I	Matrix metalloproteinase inhibitor
MMP-2I	Matrix metalloproteinase-2 inhibitor
MSH	Melanocyte stimulating hormone
MT-MMP	Membrane-type matrix metalloproteinase
MW	Molecular weight
MWCO	molecular weight cut-off
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NGM	Normal growth medium
NO	Nitric oxide
NS	Normal scar
OD	Optical density
p53	p53 transcription factor
PA	Plasminogen activator
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly(ADP-ribose)polymerase
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide-3 kinase
PMSF	Phenylmethylsulfonyl fluoride
pRB	Retinoblastoma protein–tumour suppressor protein
RAD	Arginine-Alanine-Aspartate amino acid motif
RGD	Arginine-Glysine-Aspartate amino acid motif
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SARA	SMAD anchor for receptor activation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFM	Serum free medium
Shc	Src homology and collagen protein
siRNA	Small interfering RNA
SMAC	Second mitochondria activator of caspase
T0	Time zero
TAE	Tris-acetate-ethylenediaminetetacetic acid
TdT	Terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TGF-βR	Transforming growth factor-beta receptor
TIEG	Transforming growth factor-beta-inducible early gene
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour necrosis factor-alpha
TNFR1	Tumour necrosis factor receptor-1
TRADD	TNF adaptor death domain
TRAF	TNF receptor-associated factor
TRIS	Tris[hydroxymethyl]aminomethane
tTBS	Tween-Tris buffered saline
UVP	Ultraviolet projector
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand Factor
3-D	Three-dimensional
+ve	Positive
-ve	Negative

Chapter 1

Review of the Literature

1.1 Introduction

Keloid scarring is a fibrotic condition caused by a deregulation of the wound repair process. It is a significant clinical problem as there is no reliably effective treatment or cure for this distressing condition. By gaining an understanding of the aetiology of keloid scarring, we may be able to develop an effective cure or preventative treatment. In addition, these findings may have implications for many other fibrotic conditions and provide us with a comprehensive insight into the processes that take place during normal tissue repair. Moreover, it is feasible that knowledge of this kind may even lead to us being able to manipulate the wound healing process to achieve complete tissue regeneration.

Scarless healing is known to occur in early mammalian embryos (Ferguson *et al.*, 1996), as well as in the gingival repair process (Stahl, 1976) and in some instances in the liver (Friedman and Bansal, 2006). Complete regeneration also occurs in lower vertebrates, such as Salamanders and invertebrates (Brockes *et al.*, 2001). It is proposed that the process of wound healing in skin in the post-natal mammal is optimised for survival, where a rapid, surplus, inflammatory response takes place involving numerous overlapping cytokine and inflammatory cascades, thus allowing the wound to heal quickly under non-sterile conditions and to prevent infection and future wound breakdown (Bayat *et al.*, 2003) thus:

"A scar may therefore be the price we pay for evolutionary survival after wounding"

Bayat *et al.*, 2003

In the modern world cutaneous wounds are commonplace, whether caused by surgical procedure, disease or injury, and affect people of all age groups. According to figures published in the NHS Health and Social Care Information Centre, for the financial year 2004-2005 in England alone, over 56,000 patients (6.5 patients every hour) were treated in hospital for bodily injury that resulted in an open wound. Of these wounds, over 35,000 affected the face and head. For burns injury in the same year nearly 10,000 patients were treated in A&E or specialist burns units, which is 26 a day. Appallingly, 41% of these patients were children (<14yrs), with pre-school children being at greatest risk accounting for 75% of all severe child injuries (DTI Government Consumer Safety Research document entitled "Burn and Scalds Injury in the Home").

These figures are diminutive compared to the millions per year who undergo surgical procedures in the UK, all of which result in scar formation.

Scars are often considered as trivial, but can disfigure the affected area and in instances of pathological scarring not only are these scars particularly ugly, raised and lumpy, but they can also cause severe pruritus, tenderness and pain (Bell *et al.*, 1988). Other problems associated with pathological scarring include psychosocial problems: sleep disturbance, anxiety, depression, disruption of daily activities, development of post-traumatic stress reactions, loss of self-esteem, and stigmatisation leading to a diminished quality of life (Bell *et al.*, 1988; Taal and Faber, 1998; Robert *et al.*, 1999). Physical deformity can also result from scar contractures (Figure 1.1), which can be disabling if the contracture diminishes normal movement (Woo and Seul, 2001).

Figure 1.1 Scarring after a burns injury demonstrating contractures (photographs taken from the RAFT archive of consenting patients, Mount Vernon Hospital)

There is considerable quantitative and qualitative variation in scarring potential between individuals and even within the same individual (Sommerlad and Creasey, 1978). Skin tissue repair results in a broad range of scar types, ranging from a 'normal' pale-flat scar, to abnormal scars including widespread scars, atrophic scars

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Switching off the Fibroproliferative Phase of Wound Healing – An Investigation of the Normal Mechanisms and Pathological Scar-related Defects

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A thesis submitted to the University of London for the degree of Doctor of Philosophy (Ph.D)

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Dedication

I wish to dedicate this thesis to my family. To my parents, for their continued support, love and admiration for my work. To my sister for always listening and encouraging me. Finally, to my husband for being understanding, filling me with self-confidence and supporting me both financially and emotionally.

Thanks.

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Vigor C, Rolfe KJ, Richardson J, Baker R, Grobbelaar A, Linge C. The mechanism of apoptosis induction during normal wound healing – The role of the ECM. Oral presentation, European Conference for Scientists and Plastic Surgeons, Belgium, Sept 2005.

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Vigor C, Sanders R, Linge C. The biphasic role of TGF-β1 modulates wound healing. Oral presentation, European Conference for Scientists and Plastic Surgeons Munich, Oct 2004.

C Vigor, KJ Rolfe, J Richardson, R Sanders, C Linge. The changing role of TGF-β1 during wound healing. Poster presentation, European Society of Dermatology Research, Vienna, 2004. Published *J Invest Dermatol* 2004; 123 (2): A58.

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Vigor C, Linge C, Sanders R. Aetiology of keloid scarring. Oral presentation, European Conference for Scientists and Plastic Surgeons. Geneva, Sept 2003.

Papers

Linge C, Richardson J, **Vigor C**, Clayton E, Hardas B, Rolfe K. Hypertrophic scar cells fail to undergo a form of apoptosis specific to contractile collagen - the role of tissue transglutaminase. *J Invest Dermatol.* 2005 Jul;125 (1):72-82.

Vigor C, Eade L, Linge C. Is the aetiology of keloid scarring due to an aberrant response to profibrotic growth factors, or is this phenomenon specific to all 'scar fibroblasts'? Submitted *British J Plast Surg.*

Vigor C, Richardson J, Linge C. Keloid scar cells fail to respond to the effects of collagen remodelling induced apoptosis. *In preparation.*

Vigor C and Linge C. Potential mechanisms of collagen contraction induced apoptosis – apoptosis cues during granulation tissue remodelling. *In preparation.*

Abstract

Using *in vitro* models that mimic various aspects/stages of wound healing, this thesis attempted to define the events that lead to the culmination of the fibro-proliferative phase of wound healing, i.e. apoptosis and any potential defects exhibited by keloid scars. Normal scar-derived fibroblasts were found to undergo apoptosis in contractile collagen gels, whereas keloid fibroblasts did not.

Investigation of the mechanisms involved indicated that this form of apoptosis required both the three-dimensional and collagenous nature of the gel and was not simply caused by removal of tension, but required biochemical cues. Collagen-contraction-induced apoptosis was found to require matrix metalloproteinase (MMP) and autocrine transforming growth factor-β (TGF-β) activity. Indeed contraction was accompanied by significantly increased expression and activation of MMPs along with indications of increased matrix breakdown. Furthermore, pure products of matrix breakdown significantly induced apoptosis of normal scar cell monolayers.

The defect exhibited by keloid fibroblasts was found to be specific to that induced during collagen contraction since they were equivalent to normal scar cells in their sensitivity to other forms of apoptosis induction and demonstrated normotrophic p53 stabilisation and activation of PARP (Poly(ADP-ribose)polymerase) and caspase-3. During collagen contraction, keloid fibroblasts failed to produce biochemical cues of apoptosis and although they exhibited normal levels of MMP gene expression and activation, they failed to breakdown collagen gels. However, these cells did undergo apoptosis in response to the biochemical cues produced on normal scar cell contraction of collagen gels, but surprisingly could not respond to pure forms of matrix breakdown products. Unlike normal scar cells, keloid fibroblasts failed to differentiate into myofibroblasts in collagen gels. The addition of exogenous TGF-β1 was found to restore differentiation and furthermore allowed the cells to undergo apoptosis in collagen gels. Surprisingly, TGF-β1 also restored the ability of keloid cells to undergo apoptosis in response to matrix breakdown products.

Declaration of Originality

I declare that the laboratory research for this thesis is original and that the ideas were developed in conjunction with my supervisor. I would like to thank Janette Richardson for performing the experiments for the results presented on page 108 and 255. All other experiments were performed by myself with the guidance and technical assistance of the laboratory and scientific staff at the RAFT Institute of Plastic and Reconstructive Surgery, Mount Vernon Hospital.

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Abbreviations

A	Anchored
A+	Anchored + TGF-β
ADAM	A disintegrin metalloproteinase
ADAMT	A disintegrin metalloproteinase with a thrombospondin motif
ANOVA	Analysis of variance
Anti-TGF-β	Anti-Transforming growth factor-1 antibody
APAF-1	Apoptosis protease inducing factor-1
APS	Ammonium persulfate
ARTS	Apoptosis-related protein in TGF-beta signalling pathway
α-SMA	Alpha smooth muscle actin
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C	Contractile
C+	Contractile + TGF-β
C3	Complement factor-3
C4	Complement factor-4
CAD	Caspase-activated DNase
CARD	Caspase-associated recruitment domain
Casp	Caspase
Casp-3I	Caspase -3 inhibitor
CDK	Cyclin-dependant kinase
c-FLIP	Cellular FLICE-like inhibitory protein
$CHCl_3$	Isoamylalcohol
CTGF	Connective tissue growth factor
DAPI	4',6-Diamidino-2-phenylindole
DD	Death domain
DDM	Aspartate-aspartate-methionine amino acid motif
DED	Death effector domain
DEPC	Diethylpyrocarbonate
dH_2O	Distilled water
DIABLO	Direct IAP binding protein
DISC	Death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleoside triphosphates
DPX	Dibutyl phthalate xylene
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
ECM	Extracellular matrix
ED-A Fn	EIIIA fibronectin
EDTA	Ethylenediaminetetacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signalling-regulated-kinase
FAC	Focal adhesion complex
FADD	Fas adaptor death domain

FAK	Focal adhesion kinase
FasL	FAS ligand
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GT	Guanidine thiocyanate
GTPases	Guanadine thiosulphate phosphatases
HA	Hyaluronic acid
HB-EGF	Heparin-binding epidermal growth factor
HLA-C2	Human leukocyte antigen-class 2
IAP	Inhibitor of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
ICAM-1	Intercellular adhesion molecule-1
ICE	Interleukin 1β-converting enzyme
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
IP-10	Inositol phosphatase-10
JNK	c-Jun-N-terminal kinase
KCL	Potassium chloride
KGF	Keratinocyte growth factor
KLD	Keloid
LAP	Latency-associated peptide
LPA	Lysophosphatidic acid
LTBP	Latent TGF-β-binding protein
M6P	Mannose-6-phosphate
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1alpha
MMP	Matrix metalloproteinase
MMP-I	Matrix metalloproteinase inhibitor
MMP-2I	Matrix metalloproteinase-2 inhibitor
MSH	Melanocyte stimulating hormone
MT-MMP	Membrane-type matrix metalloproteinase
MW	Molecular weight
MWCO	molecular weight cut-off
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NGM	Normal growth medium
NO	Nitric oxide
NS	Normal scar
OD	Optical density
p53	p53 transcription factor
PA	Plasminogen activator
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly(ADP-ribose)polymerase
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide-3 kinase
PMSF	Phenylmethylsulfonyl fluoride
pRB	Retinoblastoma protein–tumour suppressor protein
RAD	Arginine-Alanine-Aspartate amino acid motif
RGD	Arginine-Glysine-Aspartate amino acid motif
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
SARA	SMAD anchor for receptor activation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFM	Serum free medium
Shc	Src homology and collagen protein
siRNA	Small interfering RNA
SMAC	Second mitochondria activator of caspase
T0	Time zero
TAE	Tris-acetate-ethylenediaminetetacetic acid
TdT	Terminal deoxynucleotidyl transferase
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-α	Transforming growth factor-alpha
TGF-β	Transforming growth factor-beta
TGF-βR	Transforming growth factor-beta receptor
TIEG	Transforming growth factor-beta-inducible early gene
TIMP	Tissue inhibitor of matrix metalloproteinase
TNF-α	Tumour necrosis factor-alpha
TNFR1	Tumour necrosis factor receptor-1
TRADD	TNF adaptor death domain
TRAF	TNF receptor-associated factor
TRIS	Tris[hydroxymethyl]aminomethane
tTBS	Tween-Tris buffered saline
UVP	Ultraviolet projector
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VWF	Von Willebrand Factor
3-D	Three-dimensional
+ve	Positive
-ve	Negative

Chapter 1

Review of the Literature

1.1 Introduction

Keloid scarring is a fibrotic condition caused by a deregulation of the wound repair process. It is a significant clinical problem as there is no reliably effective treatment or cure for this distressing condition. By gaining an understanding of the aetiology of keloid scarring, we may be able to develop an effective cure or preventative treatment. In addition, these findings may have implications for many other fibrotic conditions and provide us with a comprehensive insight into the processes that take place during normal tissue repair. Moreover, it is feasible that knowledge of this kind may even lead to us being able to manipulate the wound healing process to achieve complete tissue regeneration.

Scarless healing is known to occur in early mammalian embryos (Ferguson *et al.*, 1996), as well as in the gingival repair process (Stahl, 1976) and in some instances in the liver (Friedman and Bansal, 2006). Complete regeneration also occurs in lower vertebrates, such as Salamanders and invertebrates (Brockes *et al.*, 2001). It is proposed that the process of wound healing in skin in the post-natal mammal is optimised for survival, where a rapid, surplus, inflammatory response takes place involving numerous overlapping cytokine and inflammatory cascades, thus allowing the wound to heal quickly under non-sterile conditions and to prevent infection and future wound breakdown (Bayat *et al.*, 2003) thus:

"A scar may therefore be the price we pay for evolutionary survival after wounding"

Bayat *et al.*, 2003

In the modern world cutaneous wounds are commonplace, whether caused by surgical procedure, disease or injury, and affect people of all age groups. According to figures published in the NHS Health and Social Care Information Centre, for the financial year 2004-2005 in England alone, over 56,000 patients (6.5 patients every hour) were treated in hospital for bodily injury that resulted in an open wound. Of these wounds, over 35,000 affected the face and head. For burns injury in the same year nearly 10,000 patients were treated in A&E or specialist burns units, which is 26 a day. Appallingly, 41% of these patients were children (<14yrs), with pre-school children being at greatest risk accounting for 75% of all severe child injuries (DTI Government Consumer Safety Research document entitled "Burn and Scalds Injury in the Home").

These figures are diminutive compared to the millions per year who undergo surgical procedures in the UK, all of which result in scar formation.

Scars are often considered as trivial, but can disfigure the affected area and in instances of pathological scarring not only are these scars particularly ugly, raised and lumpy, but they can also cause severe pruritus, tenderness and pain (Bell *et al.*, 1988). Other problems associated with pathological scarring include psychosocial problems: sleep disturbance, anxiety, depression, disruption of daily activities, development of post-traumatic stress reactions, loss of self-esteem, and stigmatisation leading to a diminished quality of life (Bell *et al.*, 1988; Taal and Faber, 1998; Robert *et al.*, 1999). Physical deformity can also result from scar contractures (Figure 1.1), which can be disabling if the contracture diminishes normal movement (Woo and Seul, 2001).

Figure 1.1 Scarring after a burns injury demonstrating contractures (photographs taken from the RAFT archive of consenting patients, Mount Vernon Hospital)

There is considerable quantitative and qualitative variation in scarring potential between individuals and even within the same individual (Sommerlad and Creasey, 1978). Skin tissue repair results in a broad range of scar types, ranging from a 'normal' pale-flat scar, to abnormal scars including widespread scars, atrophic scars

(depressed), scar contractures (particularly common after burns injury), hypertrophic scars and keloid scars (Figure 1.2). This project concentrates on keloid scars.

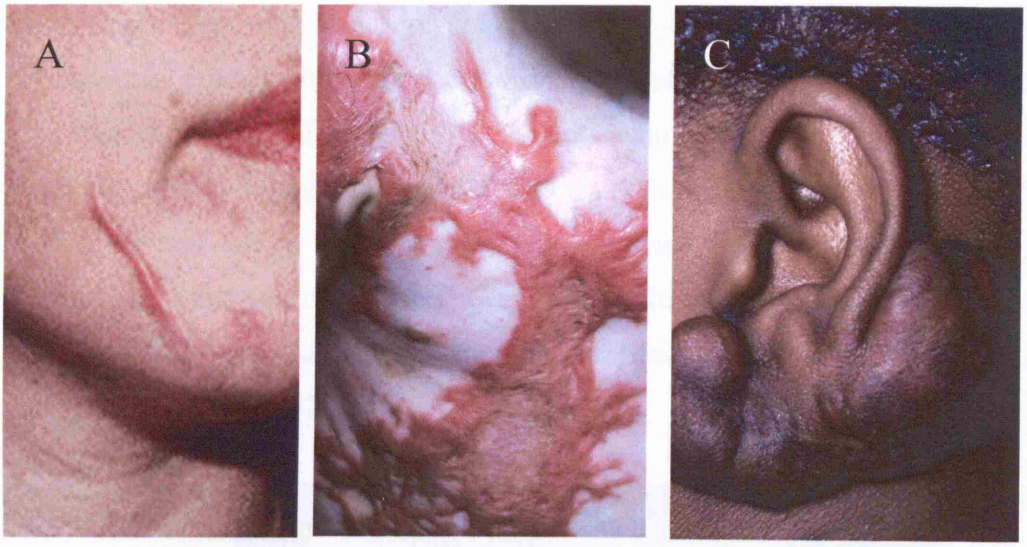

Figure 1.2. Examples of the wide range of scar types; normal (A), hypertrophic (B) keloid (C) (photographs taken from the RAFT archive of consenting patients, Mount Vernon Hospital)

1.1.1 History of Keloid Scarring

Keloids only develop in humans and as such there is no natural animal model for this condition (Rockwell *et al.*, 1989; Kischer, 1992). They occur in all races, except Albinos, with people of African, Mediterranean and Asian origin being more susceptible; developing keloids in 4.5 to 16% of all cases of cutaneous injury (Oluwasanmi, 1974; Ketchum, 1977). Keloids are mentioned throughout African folklore and their behaviour is well described in African proverbs and folk medicine (Datuba-Brown, 1990). In some rural African tribes these scars are used for decoration (Oluwasanmi, 1974; Lawrence 1987). Although this lesion was said to have been first described by Retz in the 18th century, and later by Alibert (1806); the Yorubas of Western Nigeria had depicted keloids in sculpture as early as the 13th century (Oluwasanmi, 1979). Keloids were first given the name 'cancroid' by Aliberts (1806), as they were initially thought to be a cancer-like tumour (Oluwasanmi, 1974). This was later revised to the term 'cheloid'. Cheloid is derived from the Greek word *chele,* which means crab claw, referring to the manner in which these lesions grow laterally in normal tissue (Berman and Bieley, 1995).

Fundamentally, keloids are healed human skin wounds, in which the scarring continues beyond the margins of the original wound, resembling a benign tumour (Figure 1.3) (Peacock *et al.,* 1970; Russell *et al.,* 1988). The cells are very active depositing excessive amounts of collagen (McCoy and Cohen, 1981). Keloid scar formation is gradual and can occur from 3 months to several years after trauma (Oluwasanmi, 1974; Murray, 1994). Patients who form keloid scars suffer mainly from the ugly excessive nature of these scars. Keloid scars appear as thick ugly mounds of scar tissue, which can continue growing for many years, sometimes to colossal sizes that can be crippling (Raje, 1980). In the pre-sternal area, they tend to grow in a crab-like manner and are often referred to as butterfly keloids (Abdel-Fattah, 1976; Stott, 1977).

Figure 1.3 Photographs depicting keloid scarring where the scar tissue has continued to grow excessively beyond the original confines of the wound (photographs taken from the RAFT archive of consenting patients, Mount Vernon Hospital)

1.1.2 Management of Keloids

The simplest and most direct form of treatment for keloids is by surgical excision. However, the high recurrence rates necessitate supplementary therapy attempting to inhibit either fibroblast proliferation or collagen synthesis. Different modalities such as: radiotherapy, steroids, ultrasound, cryotherapy, systemic chemotherapy and laser treatment have all been tried either alone or combined with surgery to varying degrees of success. Pharmacological agents used in the management of keloids are diverse in nature and include; bleomycin, tamoxifen, tretinion, tacrolimus, pentoxifylline,

colchicines, calcium antagonists, tranilast, vitamin E, penicillamine, retinoic acid, dextran sulphate and medacasolm (Bosse *et al.,* 1979; Janseen de Limpens, 1980; Mayou, 1981; Chiu *et al.,* 1987; Panabiere-Castaings, 1988; English and Shenefelt, 1999; Niessen *et al.*, 1999; Espana *et al.*, 2001; Kim *et al.*, 2001; Mikulec *et al.*, 2001). Again the degree of success of these treatments is varied, but none have been found to be reliably effective. In addition, unfortunately these agents are not without their complications, some causing local atrophy of tissues (Jemee, 1988) and even a systemic response (Dziewulski *et al.*, 1988).

Intra-lesional interferon injection has been found to improve keloid scars as interferon decreases the production of collagen types I and III from fibroblasts (Granstein *et al.*, 1990; Berman and Flores, 1997). The efficacy of treatment with interferon is still under debate however with varying results reported. Al-Khawajah (1996) carried out a study with 22 patients over 3 weeks, after one intralesional injection of interferon-α2b there was no significant reduction in keloid size in comparison to the dilutant alone control. Whereas, Berman and Flores, (1997) found that post operative injection of interferon-α2b (IFN-α2b) lead to only an 18.7% (3 of 16 patients) recurrence rate as compared to 51.1% of untreated control, suggesting that injection of keloid excision sites with interferon-α2b offers a therapeutic advantage. It is possible however, that different experimental designs may lead to different results; for example if interferon is given as an injection after excision of the keloid scar (as an adjuvant therapy) or as a single therapy.

Fluorouracil is also reported to improve keloid scarring in the majority of patients (Fitzpatrick, 1999; Uppal *et al.*, 2001). Uppal *et al* (2001) carried out a study on 5 patients with keloid scars, on excision of the scar a single application of 5-flourouracil solution was applied to the wound margins for 5 minutes. Their results identified a significant decrease in Ki-67 (cell proliferation marker), vascular cell adhesion molecule-1 (VCAM-1) and transforming growth factor-β1 (TGF-β1) expression in treated wounds compared to control treated wounds. In addition, functionally the fibroblasts from 3 of the 5 treated patients showed reduced contractile potential; data on keloid recurrence was not complete in this study however. Nevertheless,

prospective, well-controlled clinical studies are warranted to determine the efficacy of fluorouracil for the treatment of patients with keloid scars.

Adhesive zinc tapes and silicon gels have also been claimed to reduce keloid scars or prevent their recurrence (Soderberg *et al.,* 1982; Perkins *et al.*, 1983, 1987; Quinn *et al.*, 1985). A study by Soderberg *et al* (1982) was carried out where 41 patients suffering with keloid scars were treated with adhesive zinc tape. After 6 months, the size of the scars in 23 of the patients was reduced to the level of the surrounding skin; the remaining patients also saw a decrease in scar size. Furthermore, a number of patients found an elimination or reduction in itching. However, this trial failed to use an appropriate control by which to draw comparison. Fulton (1995), carried out a study investigating the effectiveness of silicon gel sheeting, when placed over 20 cases of evolving scars for 8-12 weeks, lesions were found to improve in 85% of the cases. Again, this study failed to use an appropriate control. In addition, this study did not determine the mode of action, they did state however that no positive pressure was needed, neither was there any silica from the dressing found at the wound site. Acrylic splints or stents have also been used in an attempt to reduce scarring; they are thought to act by exerting a positive pressure effect. However, studies do not describe whether the treatment provides a long-term cure or how effective the therapy is (Hurtado and Crowther, 1985; Pierce, 1986). Multitudes of other modalities have also been used in the treatment of keloids, however the studies are often limited by poor study design, a scarcity of subjects, and short-term follow-up. Despite these attempts there remains no reliably effective treatment of keloid scars.

To understand the pathological condition of keloid scarring an in-depth understanding of the skin and the many overlapping phases of wound healing need to be considered.

1.2 Structure and Function of the Skin

The skin is made up of three main layers: epidermis, dermis and hypodermis, consisting of heterogeneous cell types and extracellular components (Figure 1.4). This multi-layered organ also contains several specialised derivative structures known as appendages (hair follicles, eccrine sweat glands, sebaceous glands, apocrine glands).

During development and tissue homeostasis in the adult, these skin layers constantly interact with each other. The primary function of human skin is to provide the body with a protective covering (barrier function) which is relatively impermeable, mechanically strong and elastic enough to allow movement.

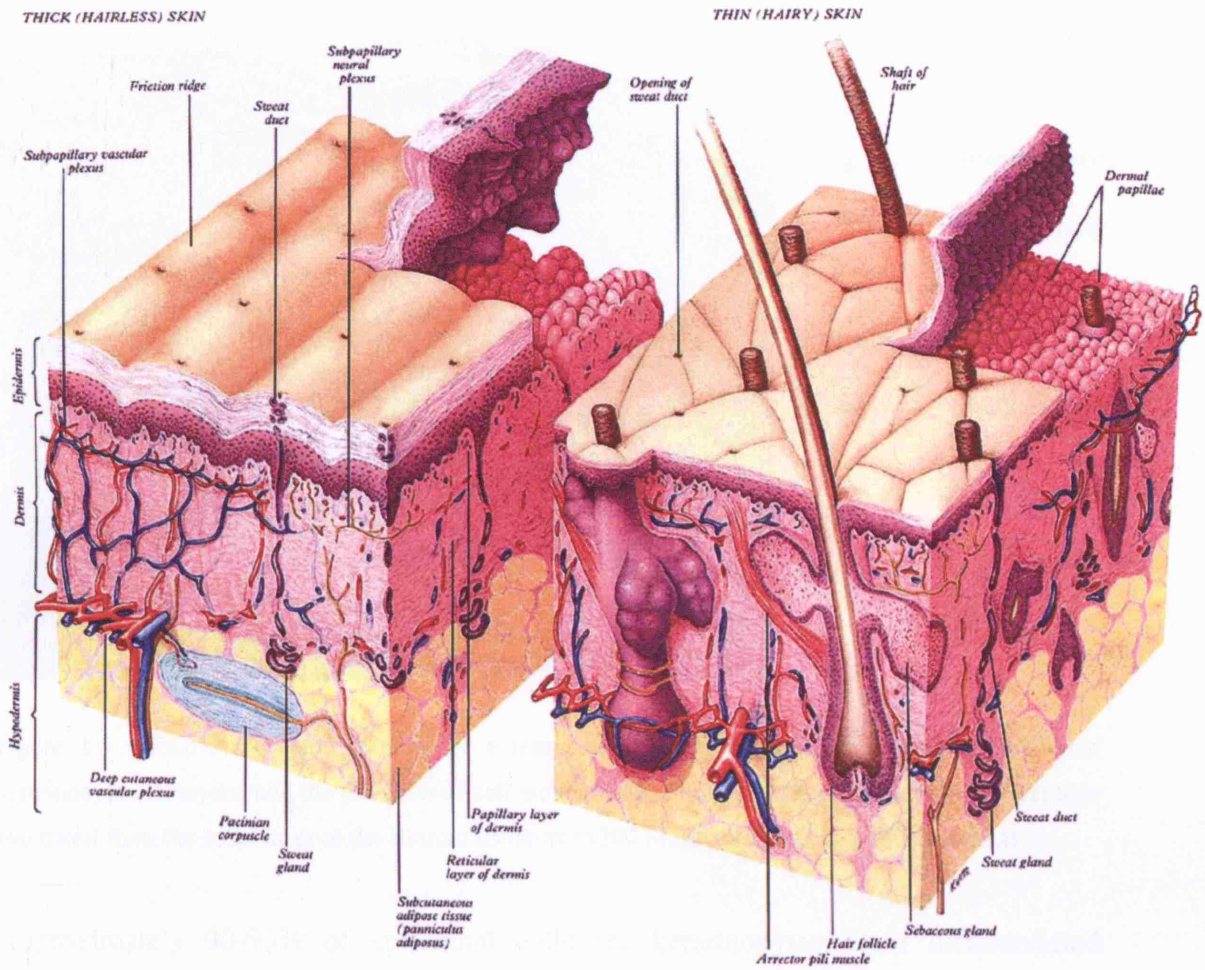

Figure 1.4. Diagrammatic representation of the skin (taken from Grays Anatomy: Williams *et al.*, 1995, 38th Edition).

The structure and function of skin and wound healing has been reviewed extensively by a number of authors (Leibovich and Ross, 1975; Postlethwaite and Kang, 1976; Agelli and Wahl, 1986; Derynck, 1988; Ross *et al.*, 1990; Brown, 1992; Damsky and Werb, 1992; Juliano and Haskill, 1993; Sporn and Roberts, 1992, 1986; Werner, 1992; Weksler, 1992; Clark, 1993a, 1993b, 1993c; Waldorf and Fewkes, 1995; Linares, 1996; Greenhalgh, 1998; Falabella and Falanga, 2001; Gordon and Chan, 2001; Haake *et al.*, 2001; Liekens *et al.*, 2001; Mauch *et al.*, 2001) A summary of these reviews is presented here with additional data cited appropriately.

1.2.1 Epidermis

The epidermis is the outermost layer of the skin and is comprised of multiple cell types derived from different embryonic origins: keratinocytes, melanocytes, Langerhans cells (antigen-presenting cells), and Merkel cells (neuroendocrine cells).

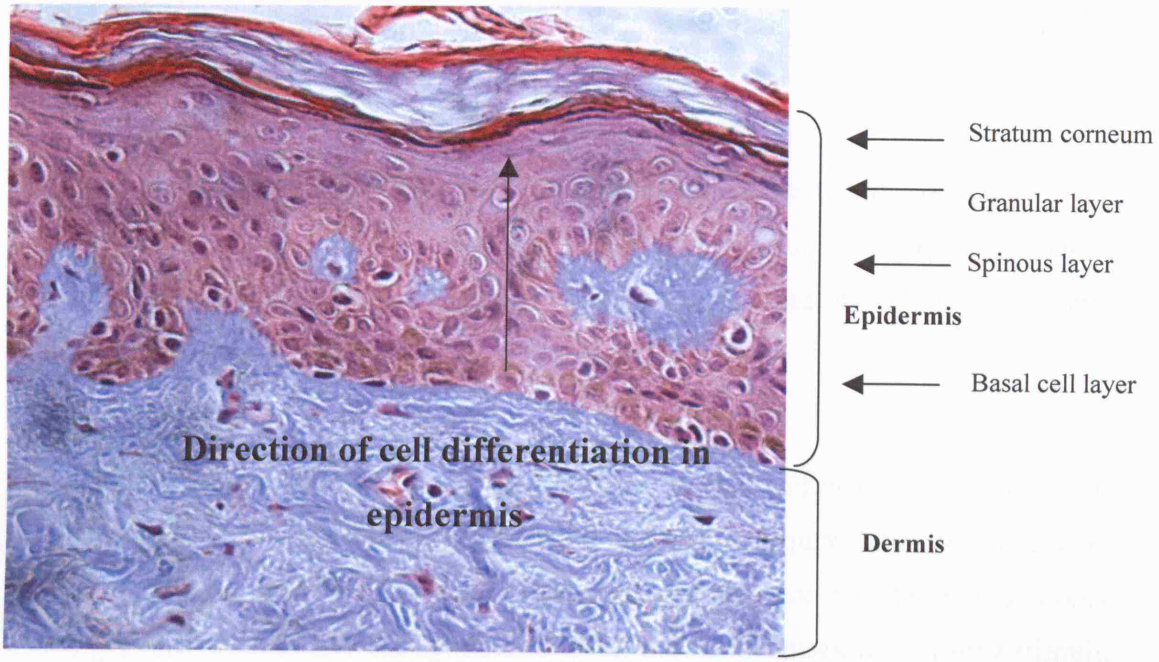

Figure 1.5 Mason's trichrome staining of a transverse section of skin, illustrating the different keratinocyte cell layers, and the direction of cell movement as keratinocytes progressively differentiate and travel from the basal layer to the stratum corneum (x200 Mag). (Vigor C, unpublished data).

Approximately 90-95% of epidermal cells are keratinocytes; their differentiated phenotype correlates with their position in the epidermis, with the most mature cells being outermost. The intermediate filament proteins known as keratins are typically found in keratinocytes, together with microfilaments and microtubules they serve a cytoskeletal role providing structural integrity. The keratinocytes within the basal layer progressively differentiate and move towards the outer skin surface (cornification) (Figure 1.5). The process of cornification is a tightly regulated series of morphological and metabolic events and involves:

1) Loss of the ability to proliferate
2) An increase in cell size and cell flattening
3) The formation of new organelles together with structural reorganisation of existing organelles, and the eventual loss of organelles
4) Synthesis of new proteins and lipids
5) Changes in plasma membrane properties, cell surface antigens and receptors
6) Dehydration

The end-point of cornification is a terminally differentiated, dead-cell (the corneocyte). Although this cell is not viable it is still considered to be functional in terms of its important barrier properties, including permeation of environmental substances and preventing water loss.

1.2.2 Dermis

The dermis is the connective tissue component of the skin and provides pliability and tensile strength. It also protects the body from mechanical injury, binds water, aids in thermal regulation, provides support for the epidermis and contains the blood supply which provides nutrients to the epidermis and contains receptors for sensory stimuli. The epidermis and dermis interact with each other during development and in the repair and remodelling of the skin after wounding. The dermis is much less cellular than the epidermis, composed of mainly fibrous extracellular matrix surrounding epidermal appendages, vascular networks, sensory receptors and dermal cells (Figure 1.4). The architecture differs throughout the dermis, with fewer fibroblastic cells being present deeper within the dermis, and deeper still there is an increase in the number of adipocytes which marks the border between dermis and hypodermis. The cells within the dermis do not undergo any obvious sequence of differentiation throughout their life span like epidermal cells; however, the matrix components do undergo turnover and remodelling in normal skin, in pathological processes and in response to external stimuli.

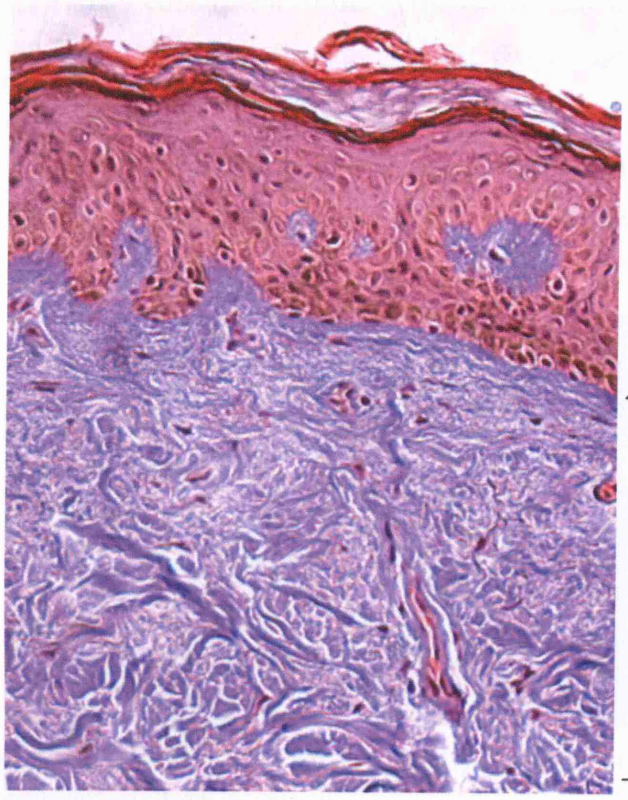

Figure 1.6. Masson's trichrome staining of a transverse section of skin to illustrate the dermal layers (x100 Mag) (Vigor C, unpublished data).

The papillary (most superficial) layer of the dermis is characterised by small bundles of small-diameter collagen fibrils and oxytalan elastic fibres (Figure 1.6). The structural characteristics of the matrix in the papillary dermis are thought to accommodate and dissipate mechanical stress. This region also has a high density of fibroblastic cells (not shown in Figure 1.6) that proliferate more rapidly, have a higher rate of metabolic activity than those in the reticular dermis, and they also synthesise different species of proteoglycans.

The reticular dermal layer in comparison is composed of mainly large-diameter collagen fibrils, organised into large interwoven fibre bundles (Figure 1.6). A superstructure is formed around the collagen fibre bundles consisting of branching elastic fibres. These two fibre systems are integrated, providing the dermis with strong resilient mechanical properties. In normal individuals the size of the collagen and elastic fibres increases progressively towards the underlying hypodermis.

The main constituent of the dermis is collagen (Jones *et al.,* 1995), of which types I, III and V account for the greatest proportion. In the adult, about 80-90% of the collagen is type I and 8-12% is type III. Type V collagen co-distributes and assembles into fibrils with both type I and III collagen; it is suggested that this assists in regulating fibril alignment. Figure 1.7 illustrates the alignment of the collagen fibrils.

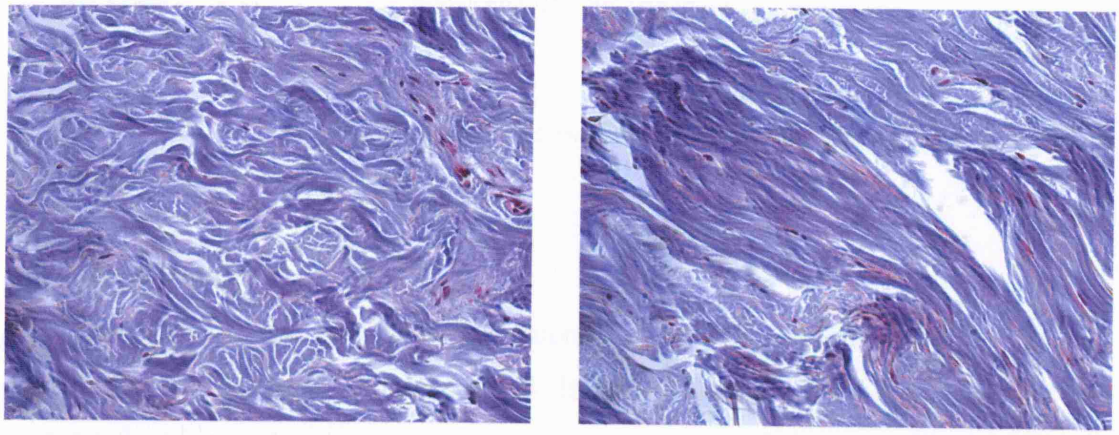

Figure 1.7 Masson's trichrome staining of the dermis, illustrating collagen fibril alignment (x200 Mag). (Vigor C, unpublished data).

The primary cell type of the dermis is the fibroblast (Figure 1.8). It is a mesenchymally-derived cell that migrates through the tissue and is responsible for the synthesis and degradation of fibrous and non-fibrous connective tissue matrix proteins and a number of soluble factors. A single fibroblast cell is capable of synthesising many different types of matrix proteins, enzymes and soluble factors. Fibroblasts interact with both resident and non-resident cells in the epidermis and dermis; fibroblasts are well integrated into the immunological microenvironment in the skin. Fibroblasts only produce multiple cytokines after activation by external stimuli, such as the inflammatory immune response, where fibroblasts are induced to express pro-inflammatory mediators to enhance the inflammatory events in the skin. With regards to wound healing, there is great interest in fibroblast regulation because of their increased proliferative and synthetic activity during the formation of scars.

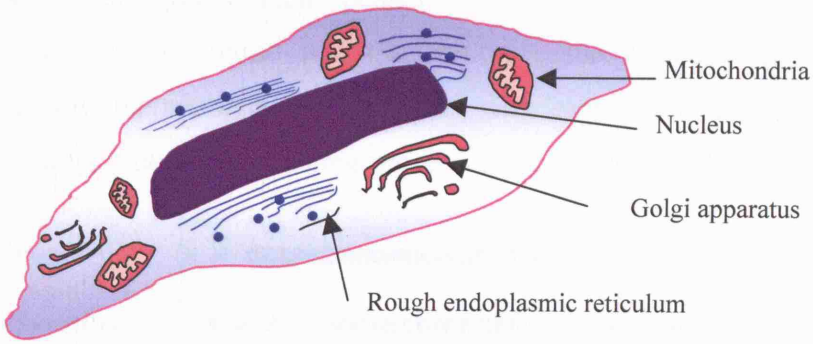

Figure 1.8 Illustration of a fibroblast recognised by the shape, vast amounts of rough endoplasmic reticulum and large prominent nucleus.

1.2.3 Hypodermis

The hypodermis is the deepest layer of connective tissue, which consists of loosely packed connective tissue and adipocytes. It acts to insulate the body, cushions and protects the skin and serves as a reserve energy supply. It has a cosmetic effect in moulding the body contours. The margin between the deep dermis and hypodermis is a sudden transition from a mainly fibrous dermal connective tissue to an adipose-rich subcutaneous tissue region. The two regions are nevertheless integrated through epidermal appendages, nerve and vascular networks (Figure 1.4).

1.3 Understanding the Normal Wound Repair Process

In higher vertebrate animals, when injury disrupts the normal architecture of the skin, it never fully regenerates. Instead a fibro-proliferative response is induced, resulting in a fibrotic scar. Alteration in the normal healing process, for example if the injury persists or recurs, can lead to continued inflammation, prolonging the repair process. There are also many pathological conditions such as: diabetes, Cushing's syndrome, poor arterial perfusion, poor nutrition and sepsis, which disrupt normal wound healing leading to non-healing wounds or excessive scarring.

Wound repair is not a simple inflexible cascade of growth factor release activating parenchymal cell proliferation and migration, but rather is a reactive process involving the interplay of soluble mediators, blood elements, extracellular matrix proteins and parenchymal cells. The wound repair process however, does follow a specific time sequence illustrated in Figure 1.9. Historically wound healing has been

separated into three main phases, for ease of discussion that is: inflammation, fibroproliferative phase and granulation tissue remodelling, with each of these processes interacting and overlapping with each other. These phases together with other important wound healing processes are described below.

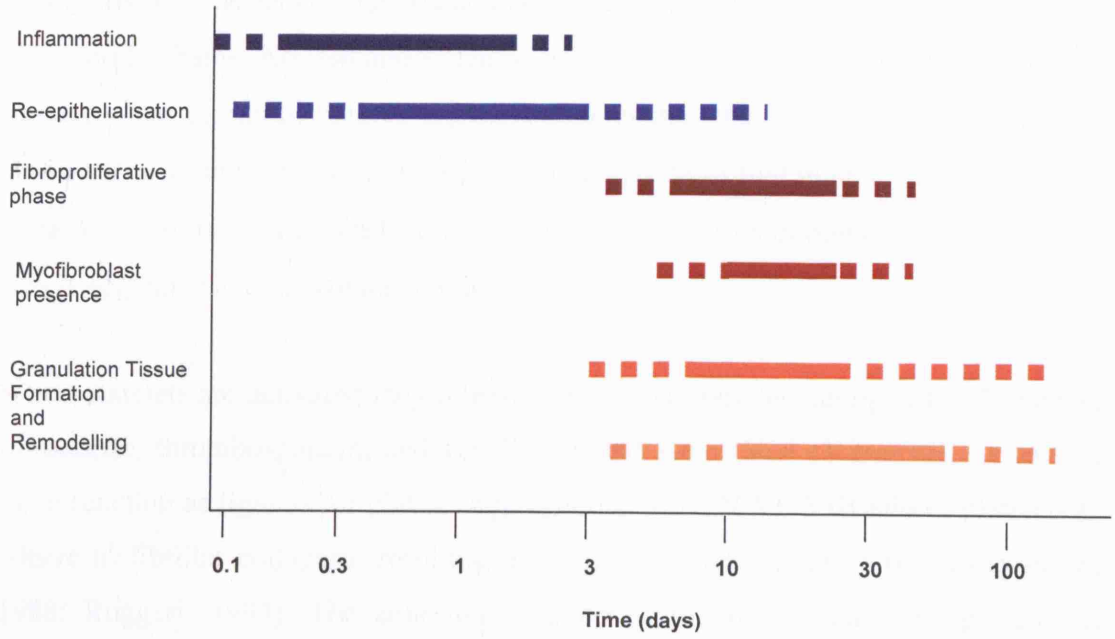

Figure 1.9 The different phases of normal wound healing (continuous line represents major activity, dotted line represents minor activity). Adapted from Clark, 1995.

1.3.1 Inflammation (Phase 1)

1.3.1.1 Blood Coagulation

Tissue injury invariably causes blood vessel disruption and subsequent release of blood constituents into the surrounding tissue. Blood coagulation and platelet aggregation generates a fibrin clot, which obstructs blood flow from severed blood vessels. Clotting results from an array of simultaneously occurring events including: activation of Hageman factor which leads to the generation of its fragments, bradykinin and potent vasoactive agents (Yamamoto and Cochrane, 1981; Muller-Esterl, 1989), release of pro-coagulant factors from damaged cells, along with surface membrane coagulation factors and phospholipids expressed on activated platelets and endothelial cells.

The major constituents of the clot are: fibrin, fibronectin, vitronectin, plasminogen, plasminogen activator, plasminogen activator inhibitor, von Willebrand factor,

thrombin and thrombospondin in addition to a variety of growth factors, cytokines and chemoattractants. This composition not only facilitates firm integration with the wound margins, but also provides a scaffold for cell migration and proliferation, along with a reservoir for growth factors and other modulators of cell function. Fibrin is the major structural protein of the clot and is derived from fibrinogen which consists of 3 polypeptide chains, Aα, Bβ and γ chains, held together by disulphide bonds. Fibrin is formed when thrombin cleaves fibrinopeptide A and then B from fibrinogen. This generates new amino-terminal ends in the α and β chains that interact with sites in the γ chain, this subsequently leads to the generation of fibrin monomers into protofibrils, which aggregate and intertwine, producing a clot.

When platelets are activated they release their alpha granules, along with fibrinogen, fibronectin, thrombospondin, and von Willebrand factor VIII (VWF-VIII). The first three function as ligands for platelet aggregation, while VWF-VIII allows platelets to adhere to fibrillar collagens, resulting in further platelet activation (Ginsberg *et al.*, 1988; Ruggeri, 1993). The adhesion of platelets to all four adhesive proteins is mediated through integrins (cell surface receptors), in particular, the platelet membrane receptor GPIIb-IIIa (αIIbβ3 integrin). This receptor has been found to bind to fibrinogen or fibrin, VWF-VIII, fibronectin and vitronectin (Ginsberg *et al.,* 1992), which facilitates coagulation and further platelet activation.

Although the major function of blood coagulation is haemostasis, it has two other functions: that of providing a provisional matrix scaffold for cell recruitment into the injured site and also having a significant role in the inflammatory response. Specifically, fibrin in conjunction with fibronectin provides a provisional matrix for the influx of monocytes (Lanir *et al.*, 1988) and fibroblasts (Grinnell *et al.*, 1980; Brown *et al.*, 1993). It is thought that migrating cells use integrin receptors that recognise fibrin, fibronectin and vitronectin to interact with the clot matrix. As fibroblasts migrate into the wound space they begin to lay down granulation tissue proteins such as cellular fibronectin and hyaluronan. In this manner the wound slowly evolves from a plasma-derived provisional matrix to a cell-derived provisional matrix. As well as promoting the migration of fibroblasts into the clot, the provisional fibrin matrix is also able to influence gene expression in these cells, for instance a fibrin

matrix reduces fibroblast collagen mRNA expression (Pardes *et al.*, 1995), but in the presence of TGF-β stimulation of fibroblasts, the fibrin matrix allows collagen synthesis. In contrast however, TGF-β leads to a down-regulation in collagen synthesis when fibroblasts are in a collagenous matrix. It is postulated that the provisional fibrin matrix promotes an early granulation tissue fibroblast phenotype, encouraging migration and proliferation, in comparison to the collagen matrix, which promotes a relatively quiescent fibroblast phenotype (Grinnell, 1994).

Initially, plasma fibronectin is deposited along with fibrin, after clot lysis cellular fibronectin begins to be deposited by a variety of wound cells such as epithelial and mesenchymal cells (Grimwood *et al.*, 1988). Fibronectin is a well-known multifunctional cell adhesion protein found in the blood in a variety of tissues and is present within clots at significant concentrations. It is encoded by a single gene, but exists in a number of variant forms that differ in sequence at three general regions of alternative splicing. However, it is nearly always composed of two identical chains linked by a single disulfide bond (Figure 1.10). Fibronectin contains specific functional domains and cell-binding sites that allow interaction between a wide range of cell types, ECM components and cytokines.

Concomitant with the release of matrix scaffold proteins, platelets also release chemotactic factors for the recruitment of leukocytes, as well as growth factors which encourage tissue regeneration such as: platelet-derived growth factor (PDGF), transforming growth factor-alpha (TGF-α) and –beta (TGF-β), epidermal growth factor (EGF) and insulin-like growth factor (IGF) (Table 1.1).

Blood clotting itself is able to contribute to the inflammatory response through the activation of Hageman factor, which not only aids clot formation but also initiates the start of the classical and alternative complement cascades (Ghebrehiwet *et al.*, 1981; DiScipio, 1982), resulting in the generation of anaphylatoxins C3a and C5a. The anaphylatoxins directly increase blood vessel permeability and attract neutrophils and monocytes to sites of tissue injury (Fernandez *et al.*, 1978).

1.3.1.2 Leukocytes

The migration of neutrophils and monocytes into the injured tissue is a consecutive process, via chemotactic gradients, where neutrophils are first to reach to wound site due to their abundance in circulation (Ross, 1968). Chemoattractants for leukocyte migration include fibrinopeptides cleaved from fibrinogen by thrombin, fibrin degradation products produced by plasmin degradation of fibrin, C5a from the activated classical or alternative complement cascades, leukotriene B_4 released by activated neutrophils, platelet activating factor (PAF) released from endothelial cells or activated neutrophils, PDGF and platelet factor 4 released from platelets (Tonnesen *et al.*, 1984).

The role of neutrophils in wound repair is to efficiently clear the wound-site of any foreign substance, including bacteria. When overwhelmed by the level or nature of contaminants, neutrophils can cause additional damage as they attempt to clear the wound, through the release of enzymes and toxic oxygen products. Once the wound has been cleared, the release of leukocyte chemoattractants usually ceases. Any remaining neutrophils in the wound area are either phagocytosed by macrophages or fibroblasts or extruded with the eschar or scab.

Monocyte accumulation often continues after neutrophils have disappeared from the wound. Their migration is stimulated by specific chemoattractants that include: fragments of collagen, elastin (Senior *et al.*, 1980), fibronectin (Clark *et al.*, 1988), enzymatically-active thrombin (Bar-Shavit *et al.*, 1983), and TGF-β. Once in the tissue, monocytes progressively become activated and differentiate into macrophages. Like platelets, macrophages release growth factors that initiate granulation tissue formation. However, unlike platelets, which simply release stored proteins and peptides, macrophages continually synthesise and secrete these factors. Macrophages produce TGF-β, PDGF, TGF-α, IGF-1, interleukin-1 (IL-1) and fibroblast growth factor (FGF) (Table 1.1). Macrophage activity is required for the generation of new tissue in wounds, since macrophage depleted animals exhibit delayed fibrin clearance and defective granulation tissue formation during wound repair (Liebovich and Ross, 1975). In this manner, macrophages appear to play a role in both inflammation and granulation tissue formation.

Table 1.1 Growth Factors Involved in Wound Repair.

Growth Factor	Effects	Cell of origin
Basic-fibroblast growth factor and acidic-fibroblast growth factor	Fibroblast and epidermal cell proliferation, angiogenesis, mitogenesis, chemotaxis	Monocytes, macrophages, endothelium
Insulin-like growth factor-1 and -2 (IGF)	Stimulates proliferation of fibroblasts and endothelial cells, chemotaxis	Fibroblasts, platelets
Keratinocyte growth factor (KGF)	Keratinocyte proliferation	Fibroblasts
Platelet-derived growth factor-AA, -AB and –BB (PDGF-AA, -AB -BB)	Fibroblast chemotaxis, proliferation and contraction, increased adhesion molecules, myofibroblast differentiation, upregulate protease production, stimulates production of collagenase by fibroblasts	Endothelial cells, platelets, macrophages, fibroblasts, monocytes, smooth muscle cells
Transforming growth factor-alpha (TGF-α)	Pleiotropic effects on cell motility and proliferation	Macrophages, eosinophils, keratinocytes, platelets
Epidermal growth factor (EGF)	Stimulates proliferation of fibroblasts, keratinocytes and endothelial cells	Monocytes, macrophages, platelets
Transforming growth factor-beta 1 and 2 (TGF-β)	Fibroblast chemotaxis, extracellular matrix deposition, protease inhibitor secretion, wound contraction, increased adhesion molecules, myofibroblast differentiation	Macrophages, lymphocytes, fibroblasts, bone cells, keratinocytes, platelets
Transforming growth factor-beta 3 (TGF-β)	Involved in scarless wound healing, cessation of matrix deposition	Macrophages, lymphocytes, fibroblasts, bone cells, keratinocytes, platelets
Vascular endothelial growth factor (VEGF)	Vascular permeability, angiogenesis	Endothelial cells, keratinocytes, mesenchymal and stromal cells.
Interleukin-1 (IL-1)	Inhibits cell proliferation, down-regulates collagen synthesis	Monocytes, macrophages, dendritic cells, keratinocytes, langerhans cells, melanocytes fibroblasts and endothelial cells
Tumour necrosis factor-α (TNF-α)	Stimulates macrophage production of proteases, increases vascular permeability, inhibits collagen synthesis	Neutrophils, fibroblasts

1.3.2 Re-establishing the Epidermal Barrier

Within hours of injury, the intact epidermis at the wound margins forms a migrating and eventually proliferating tongue of epithelial cells, in order to cover the exposed area and restore the functional integrity of the tissue. Re-epithelialisation is critical to optimal wound healing not only because of reformation of the cutaneous barrier, but also because of its role in wound contraction.

Keratinocytes at the wound margins migrate across the open wound bed in response to chemoattractants; this is made possible by their ability to detach from the basement membrane through their secretion of matrix metalloproteinases (MMPs) and plasmin. To enable their migration, epithelial cells undergo marked phenotypic alteration. This includes retraction of intermediate filaments (keratin) of the cytoskeleton, dissolution of intercellular desmosomes and the hemi-desmosomes that anchor the cells to the basement membrane and finally formation of peripheral cytoplasmic actin filaments (Odland and Ross, 1968; Gabbiani *et al.*, 1978). The migration of epithelial cells is found to be independent of their proliferation (Winter, 1972); where basal cell keratinocyte proliferation is thought to be mediated by the local release of growth factors, along with an up-regulation of growth factor receptors, matrix components, together with loss of cell-cell contact.

TGF-β1 is known to stimulate the migration of keratinocytes *in vitro*, (Shipley *et al.*, 1986; Hebda, 1988) possibly by integrin regulation or provisional matrix deposition (Wikner *et al.*, 1988). In contrast however, TGF-β1, and -β2 are potent inhibitors of keratinocyte proliferation *in vitro* with the Smad3 pathway implicated as the negative modulator (Ashcroft *et al.*, 1999). The factors thought to be responsible for the induction of keratinocyte proliferation during re-epithelialisation include the epidermal growth factor (EGF) family; especially TGF-α (Barrandon and Green, 1987), heparin-binding epidermal growth factor (HB-EGF) (Higashiyama *et al.*, 1991), and the FGF family (O'Keefe *et al.*, 1988). These growth factors are released not only by keratinocytes themselves, but also by mesenchymal cells and macrophages (Coffey *et al.*, 1987).

Numerous animal models in which cytokine genes have been deleted or over-expressed have provided further evidence that such factors are involved in the process of epithelialisation. For instance, by inducing the expression of a dominant-negative keratinocyte growth factor (KGF) receptor in basal keratinocytes of transgenic mice, Werner *et al* (1994) found that upon skin injury the proliferation of the epidermal keratinocytes was reduced, epidermal atrophy was detected as well as abnormalities in the hair follicles, dermal hyper-thickening and substantially delayed re-epithelialisation of the wound.

Once contact is established between opposing keratinocyte sheets, mitosis and migration stop, and the cells subsequently differentiate into a stratified squamous epithelium above a newly formed basement membrane.

1.3.3 Fibroproliferative Phase (phase 2)

This phase is characterised by a substantial increase in the cellularity (fibroblast migration and proliferation) and protein production within the damaged tissue underlying the renewing epithelium. As mentioned earlier, the provisional (clot) matrix within the wound consists of fibrin, fibronectin, vitronectin, VWF-III and thrombospondin in addition to a variety of growth factors, cytokines and chemoattractants. The majority of these factors contribute towards the development of granulation tissue by encouraging the migration, proliferation and activation of quiescent fibroblasts, monocytes/macrophages and endothelial cells (angiogenesis - the generation of new capillary blood vessels from pre-existing vasculature to provide nutrients and oxygen) into the provisional wound scaffold. As discussed in section 1.3.1.2 the macrophages continually secrete cytokines necessary for further stimulation of fibroblasts and new blood vessel formation.

1.3.3.1 Fibroplasia

Once the fibroblasts have migrated into the tissue they switch their major function from migration to protein synthesis, with the cells exhibiting abundant rough endoplasmic reticulum and Golgi apparatus. In addition, during the second and third week of healing, fibroblasts differentiate into myofibroblasts, a fibroblast phenotype with characteristics of smooth muscle cells such as large bundles of α-smooth muscle

actin filaments (αSMA) deposited along the cytoplasmic face of the plasma membrane and the establishment of cell-cell and cell-matrix linkages (Welch *et al.*, 1990) (see section 5.1 for further detail). The appearance of myofibroblasts has been postulated to relate to the start of connective tissue compaction and the contraction of the wound. At this point the provisional wound matrix begins to be replaced by extracellular matrix proteins mainly secreted by the recruited fibroblasts.

Initially, mainly cellular fibronectin and hyaluronan is laid down. Fibronectin provides a provisional matrix scaffold for the migration and in-growth of cells, a linkage for myofibroblasts to affect wound contraction, and a site for collagen deposition (McDonald *et al.*, 1982). In addition, the 250-350kDa fibronectin fragment is able to support fibroblast, keratinocyte and endothelial cell adhesion and movement (Grinnell and Feld, 1979; Hsieh and Chen, 1983) through an Arg-Gly-Asp (RGD) cell binding motif (Figure 1.10), which has been found to be chemotactic for fibroblasts, endothelial cells, and monocytes when in its fragmented (120kDa) form (Postlethwaite *et al.*, 1981; Bowersox and Sorgente, 1982; Doherty *et al.*, 1990). Furthermore, fibronectin can activate macrophages and modify extracellular matrix debris making the particles more susceptible to the action of phagocytes, thus optimising the clearance of the wound site (Pommier *et al.*, 1983; Brown and Goodwin, 1988).

Fibronectin can act through a variety of integrin receptors, possibly modulating gene expression, similar to fibrin (Xu and Clark, 1996). For example, fibroblasts which adhere to the RGD cell-binding motif on the 120kDa fibronectin fragment, but not other integrin recognition sites, express metalloproteinase-1; the classic mammalian collagenase. However, when fibroblasts bind to the α4β1 integrin recognition site, CS-1, on intact fibronectin, collagenase is not induced (Huhtala *et al.*, 1995). This therefore suggests that fibronectin transmits different signals to cells depending on many factors: whether it is intact or fragmented, which binding sites are available and cellular integrin expression.

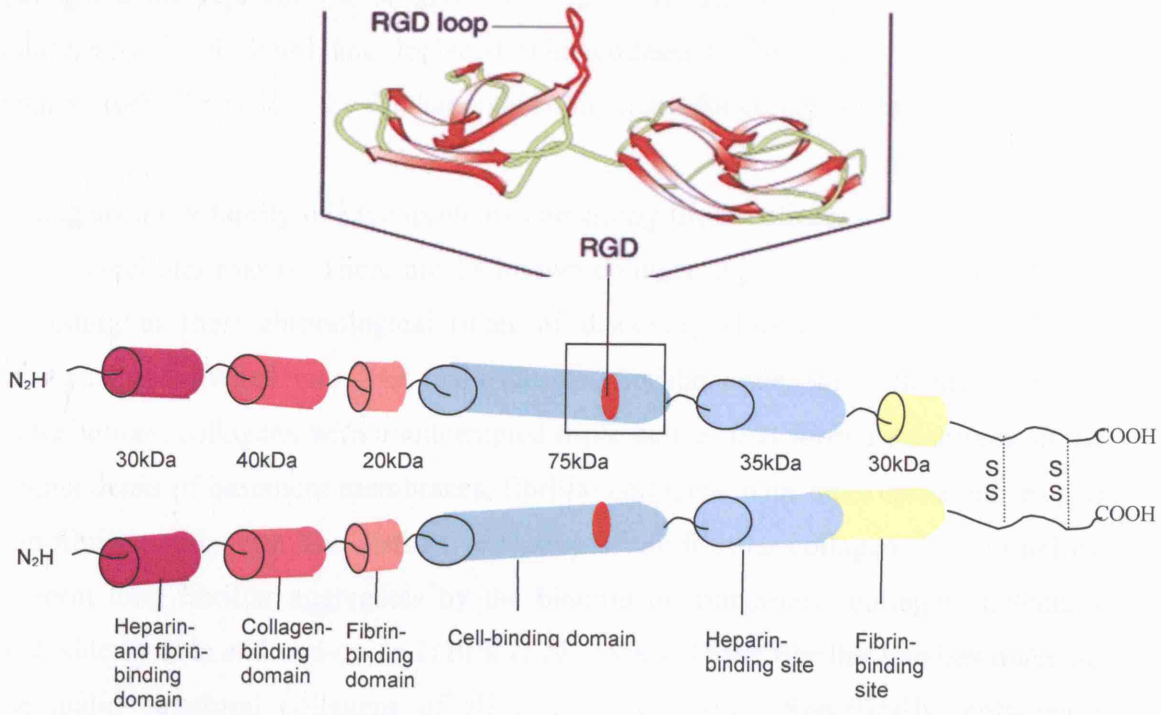

Figure 1.10 Fibronectin is constructed of two polypeptide chains associated by a disulphide bond with cell-binding sites and functional domains (adapted from Potts and Campbell, 1994).

Along with fibronectin, the initial presence of large quantities of highly hydrated hyaluronic acid (hyaluronan) in the provisional matrix allows for the easy in-growth of parenchymal cells. Hyaluronan is a linear polymer of repeating *N*-acetyl glucosamine-gluronic acid disaccharides in the general class of polysaccharides termed glycosaminoglycans. The concentration of hyaluronan is known to increase during fibroblast proliferation, suggesting that the presence of hyaluronan in the extracellular matrix may be important for cell division (Tomida *et al.*, 1974; Moscatelli and Rubin, 1975; Lembach, 1976; Hopwood and Dorfman, 1977).

As granulation tissue matures the level of hyaluronan is decreased by the action of tissue hyaluronidase (Bertolami and Donoff, 1982). The sulphated glycosaminoglycans that replace hyaluronan are associated with a protein core and are called proteoglycans. These substances give the wound increased tensile strength making the tissue more resilient, but reduce the ability of cells to move and proliferate.

Alongside the replacement of glycosaminoglycans, the fibrin/fibronectin matrix is continually being lysed and replaced with collagens. Essentially, the provisional matrix scaffold provides the fundamental components for collagen fibrillogenesis.

Collagens are a family of glycoproteins containing triple helices, which are found in the extracellular matrix. There are 18 known collagen types at present, type I-XVIII according to their chronological order of discovery (Fukai *et al.*, 1994). These collagens are divided into four main classes; fibrillar collagens with uninterrupted triple helices, collagens with uninterrupted triple helices that form a meshwork in the lamina densa of basement membranes, fibrillar collagens with interrupted helices and non-fibrillar collagens. The distinctive feature of the fibrillar collagens is their ability to form long fibrillar aggregates by the binding of monomeric collagen molecules both side-by side and end-on-end (Birk *et al.*, 1990). These fibrillar bundles make up the major structural collagens of all connective tissues. Specifically, collagen I consists of 3 coiled subunits composed of two α1(I) chains and one α2(I) chain. Collagen fibrils are formed by the intertwining of collagen α-chains into triple helices, these helices then bind to each other to form collagen fibrils, groups of which lead to collagen fibre formation (Figure 1.11).

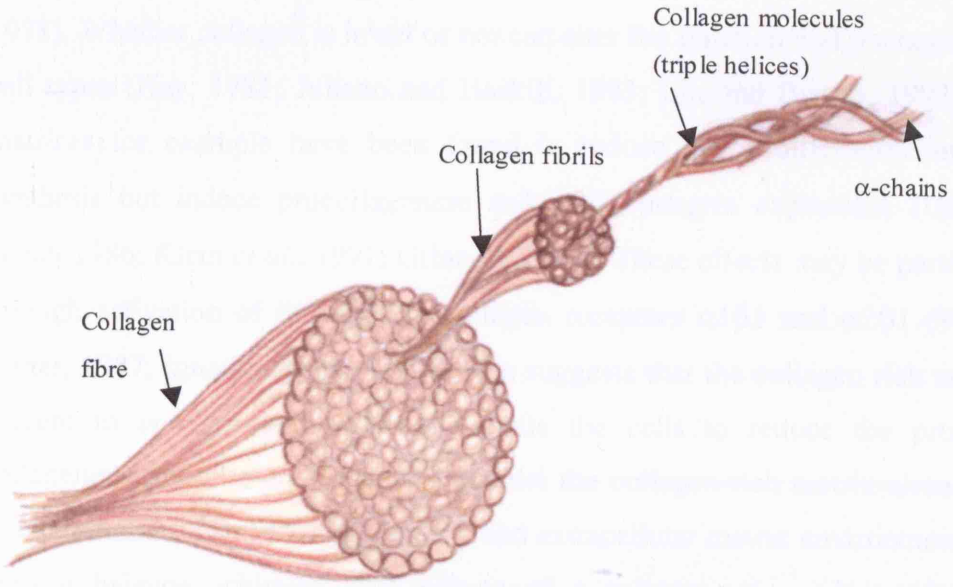

Figure 1.11 Illustration of collagen molecules forming collagen fibres (Adapted from Bhattacharjee and Bansal, 2005).

Granulation tissue was found to contain a collagen type distinct from normal adult dermis and similar to that found in embryonic skin (Bazin and Delaunay, 1964). Later, this was determined to be collagen type III (Epstein, 1974). Collagen type III does occur in small amounts in adult dermis however, it is markedly increased in granulation tissue (Gabbiani *et al.*, 1976). The evolution of granulation tissue is found to take place in the sequence of: fibronectin, collagen type III and then collagen type I (Kurkinen *et al.*, 1980).

Approximately 3 days after the wound repair process has started, increased collagen type III synthesis begins, and by day 6-7 collagen type I synthesis starts (Diegelmann *et al.,* 1975; Gabbiani *et al.*, 1976). A similar time course is also detected for types I and III collagen mRNA expression (Scharffetter *et al.*, 1989; Oono *et al.*, 1993). Type V collagen also increases during granulation tissue development along with tissue vascularity, this suggests a link between capillary endothelial cells and type V collagen (Hering *et al.*, 1983). Hypertrophic scars, another form of pathological excessive scarring, have abundant type V collagen as well as numerous capillaries providing further evidence for this link (Ehrlich and White, 1981).

As well as providing structural support for the wound, collagen-derived peptides act as chemoattractants *in vitro* and may have similar effects *in vivo* (Postlethwaite *et al.*, 1978). Whether collagen is intact or not can alter the function and phenotype of many cell types (Hay, 1981; Juliano and Haskill, 1993; Lin and Bissell, 1993). Collagen matrices for example have been found to reduce cell proliferation and collagen synthesis but induce procollagenase and $\alpha 2\beta 1$ integrin expression (Unemori and Werb, 1986; Klein *et al.*, 1991; Grinnell, 1994). These effects may be partly mediated through activation of the integrin collagen receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Wayner and Carter, 1987; Ignatius *et al.*, 1990). This suggests that the collagen rich environment present in late granulation tissue signals the cells to reduce the production of collagenous material and begin to remodel the collagen-rich matrix already present. The communication between the cells and extracellular matrix environment continues until a balance achieved. The effects of a collagenous matrix environment on fibroblasts is further considered and investigated in Chapter 3.

The incorporation of types I, III and V collagens into macromolecular structures provides tensile strength for the wound. As the wound matures, collagen bundles grow in size, proteoglycans are deposited and there is an increase in wound rigidity and resilience to deformation.

In essence, the evolving granulation tissue self perpetuates its own formation and maturation by providing a scaffold for contact guidance (fibronectin and collagen), low resistance for cell mobility (hyaluronic acid), and a reservoir for cytokines (Nathan and Sporn, 1991).

1.3.3.2 Angiogenesis

The metabolic requirements of this fast forming tissue are met by the formation of a new blood supply. Within granulation tissue, angiogenesis is potentiated by: hypoxia, nitric oxide (NO), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) (Conway *et al.*, 2001), and by the chemokines: monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein (MIP-1α) (Belperio *et al.*, 2000). VEGF is recognised as the main stimulant of endothelial cell proliferation and increases vascular permeability, it is released from wound epithelium and from the extracellular matrix by endothelial-derived proteases. Besides VEGF, FGFs act via four protein tyrosine kinase receptors to recruit endothelial cells, and also direct their proliferation, differentiation and plasminogen activator synthesis (Ornitz *et al.,* 1996). Angiogenesis is a complex process involving many factors, and as well as the modulators mentioned above, it is also affected by TGF-β1, EGF, TGF-α, endothelin 1, leptin, and indirectly by TNF-α and IL-1β.

Angiogenesis is tightly regulated by endogenous inducers and inhibitors, which mediate a decline in the process as the granulation tissue matures and scar remodelling continues. Among the identified endogenous inhibitors of re-vascularisation are: thrombospondin (TSP-1), interferon-gamma (IFN-γ), inositol phosphate-10 (IP-10), IL-12, IL-4 and the tissue inhibitors of metalloproteinase's (TIMPs), as well as the recently recognized activities of angiostatin and endostatin.

1.3.3.3 Cessation of the Fibroproliferative Phase

Once the wound site is filled with granulation tissue and covered with a new epidermis the healing process undergoes a transition between the fibroproliferative phase and the remodelling phase of wound healing. At this point the activated cells within the wound begin to become redundant and are signalled to undergo apoptosis. Studies have found that endothelial cells are the first to undergo apoptosis, followed by myofibroblasts, gradually leading to a relatively acellular scar (Compton *et al.*, 1989). The apoptosis induction mechanisms involved in signalling the removal of cells from the wound site are undefined at present. Numerous apoptosis mechanisms are potentially involved and are discussed in greater detail in section 1.7 and Chapter 3.

1.3.4 Extracellular Matrix Remodelling

(Taken from reviews by: Parsons *et* al., 1997; Massova *et* al 1998; Ravanti and Kahari, 2000; Visse and Nagase, 2003).

The remodelling of granulation tissue into a mature scar relies on the strict balance between both extracellular matrix synthesis and degradation. As the wound repair process progresses, the matrix environment changes from that of a mainly fibrin to a mainly collagen type I matrix. The degradation of wound collagen is controlled by a variety of extracellular matrix degrading enzymes, namely matrix metalloproteinases (MMPs) secreted from: granulocytes, macrophages, epidermal cells and fibroblasts. Each protease possesses overlapping specificity for different extracellular matrix substrates (Table 1.2). There are over 20 known MMPs, subdivided into 6 groups; these are discussed in further detail in section 4.1.

MMPs not only degrade matrix components, but they also function as regulatory molecules by driving enzyme cascades and processing cytokines, matrix and adhesion molecules to generate biologically active fragments. Thus the action of MMPs can alter both a cell's phenotype and behaviour, demonstrating the highly complex role of these enzymes.

Tissue inhibitors of metalloproteinases (TIMPs) tightly regulate the activity of MMPs during development and wound repair (Brenner *et al.*, 1989). TIMPs provide a natural

counterbalance to the MMPs and disruption of this balance can lead to excess or insufficient matrix degradation, suggested to result in tissue pathology (Birkedal-Hansen, 1995). Cytokines such as TGF-β, PDGF, and IL-1 and the extracellular matrix have all been found to play an important role in controlling TIMP and MMP expression *in vivo* (Werb *et al.*, 1990; Circolo *et al.*, 1991). TIMPs are also discussed further in section 4.1

Table 1.2. Matrix metalloproteinases and their substrate specificities

MMP	Alternative Name	Major Substrates
MMP-1	Collagenase-1	Collagens I, II, III, VII, VIII, X, gelatin
MMP-2	Gelatinase-A	Collagens I, II, III, IV, V, VII, X, elastin fibronectin, gelatin
MMP-3	Stromelysin-1	Collagens III, IV, V, X, IX, MMP-1, fibronectin, gelatin, laminins, proteoglycans, elastin, fibrin, pro-MMPs
MMP-7	Matrilysin	Collagen IV, elastin, fibronectin, gelatin, laminins, tenascin, processes cell surface molecules: defensin, TNF-α and E-cadherin
MMP-8	Neutrophil Collagenase	Collagens I, II, III
MMP-9	Gelatinase-B	Collagens IV, V, elastin, gelatin
MMP-10	Stromelysin-2	Collagens III, IV, V, IX, fibronectin, gelatin, laminins
MMP-11	Stromelysin-3	Fibronectin, laminins,
MMP-12	Macrophage Metalloelastase	Elastin, fibrinogen, fibronectin
MMP-13	Collagenase-3	Aggrecan, collagens I, II, III, gelatin
MMP-14	MT1-MMP	Collagens I, II, III, fibronectin, pro-MMP-13, pro-MMP-2, proteoglycans,
MMP-15	MT2-MMP	Pro-gelatinase-A
MMP-16	MT3-MMP	Collagen III, gelatin, fibronectin

1.3.5 Wound Contraction

The contacts established between cells and extracellular matrix provides a network across the wound-bed, which allows the traction of fibroblasts on their surrounding matrix to be transmitted across the wound (Singer *et al.,* 1984). Wound contraction, is thought to be carried out by the actin-rich myofibroblasts, which are most numerous in late granulation tissue and are aligned along lines of greatest tension. In a study carried out by Hinz *et al* (2001), the degree of contraction was measured of collagen matrices seeded with cells transfected with cytoplasmic actin in comparison to those transfected with α-SMA. Contraction was significantly increased in the latter, suggesting that the α-SMA isoform is instrumental in force generation by myofibroblasts.

The biomechanics of extracellular matrix contraction is found to involve F-actin bundle arrays, cell and matrix linkages and collagen cross-links (Tomasek, 1999). Collagen matrix contraction is also controlled by cytokine signals, of which TGF-β and PDGF stimulation is involved in activating fibroblasts to carry out the contraction of granulation tissue (Younai *et* al., 1996), presumably through increased α-SMA expression. Wound contraction and models of cellular contraction are detailed further in section 3.1, whereas the myofibroblast is further detailed in section 4.1.

Wounds never reach the same tensile strength as uninjured skin; at best a scar is only 70% as strong as intact skin (Levenson *et al.*, 1965). At approximately 3 weeks after injury, a wound will have only gained 20% of its final strength. Tensile strength is gradually gained by further collagen remodelling with the formation of larger collagen bundles and an alteration of inter-molecular cross-links (Bailey *et al.*, 1975).

1.4 The Aetiology of Keloid Scarring

In order to minimise scar formation the inciting agent needs to be quickly removed along with efficient culmination of the inflammatory response. Continued aggravation of the wound site leads to an incessant inflammatory response, increased localised damage, delayed healing and often increased fibrosis. Although fibrosis (continued tissue formation or fibroproliferative phase) of the skin results in scarring and disfigurement, continued deposition of matrix in internal organs such as lungs, liver,

kidney or brain not only compromises their structure, but also their function, causing disease and sometimes death. This project concentrates on the formation of keloid scars, thought to occur through continued tissue formation.

Keloid scarring has been studied over many decades, yet relatively little is known about its aetiology. What is known about pathological scarring in general has been extensively reviewed and an outline of facts relevant to keloid scarring from these reviews follows (Koonin, 1964; Sivanantharajah, 1969; Ramakrishnan *et al.,* 1974; Ketchum, 1977; Cohen *et al.*, 1979; Kurkinen *et al.*, 1980; 1988; Kazeem, 1988; Rockwell *et al.*, 1988; Babu *et al.*, 1989; Kischer *et al.*, 1989; Ford and Widgerow, 1990; Datubo-Brown, 1990; Leflore and Antoine, 1991; Buchwald *et al.,* 1992; Oliver *et al.*, 1992; Mullaney *et al.,* 1995).

In patients predisposed to keloid scarring, keloids may occur after minimal dermal injury, for example, after ear piercing, insect bites or vaccinations, whereas normal scars do not often form after injuries of these types. The susceptibility of different body regions is still unexplained: the anterior chest, shoulders, earlobes, upper arms, and cheeks have a higher predilection for keloid formation, whereas eyelids, genitalia, palms, soles, cornea, and mucus membranes and even the umbilical cord are less affected. A person with normal scarring in some areas of the body may form keloids in skin under tension, furthermore an excised keloid grafted onto an area of relatively little tension will atrophy (Calnan and Copenhagen, 1967). This phenomenon suggests that increased skin tension may possibly contribute to abnormal scarring. However, this event may involve factors other than tension since earlobes, which are free of tension, frequently develop keloids. Keloids are not limited to the skin however; keloids of the cornea have also been described (Shukla *et al.*, 1975; LeMasters and Notz, 1986).

Individuals more prone to keloid scarring have been found to be darkly pigmented African tribes with Caucasians and albinos being least affected. This has led to the suggestion that an aberration in the metabolism of melanocyte-stimulating hormone (MSH) may be responsible, although a genetic component is now considered more likely. Both sexes form keloids equally within the same age group. Keloids are however, uncommon at the early and later stages of life. This may be linked with the

fact that wound healing takes place at a slower rate in the elderly (Staley and Richard, 1993).

The incidence of keloids is also higher during times of physiologic hyperactivity of the pituitary as they often appear during puberty, resolve after menopause and enlarge during pregnancy; thus suggestive of a hormonal involvement (Moustafa *et al.*, 1975). Patients with acne keloids have a significantly higher serum testosterone, this may be linked to the origin of the disease or alternatively, it could be related with the keloid itself, as keloids show a high level of testosterone-binding receptors (George *et al.*, 1993; Schierle *et al.*, 1997).

1.4.1 Histopathology

Histologically, keloid scars are composed of tightly packed disorganised collagen fibrils; these are nodular in arrangement and are surrounded by an elevated density of activated fibroblasts or myofibroblasts and leukocytes (Figure 1.12A). This is in comparison to normal scar tissue, which appears very acellular, with loosely aligned collagen fibrils (Figure 1.12B). In addition to the increased thickness of the dermis, keloids show an increased thickness of the epidermis and a lack of rete ridges compared with normal skin (Linares *et al.*, 1972; Andriessen *et al.*, 1998).

1.4.2 Analysis of the Literature on the Nature of Keloid Scars

The nature of keloid scars has been broadly reviewed (Omo-Dare, 1975; Lewis and Sun, 1990; Tredget *et al.*, 1997; Marneros *et al.*, 2001). An overview of these reviews is presented here with supplementary data cited suitably.

1.4.2.1 Genetics

The formation of keloids is found to run in families with an autosomal recessive (Omo-dare, 1975) or dominant (Bloom, 1956) transmission, however this is not true of all cases. In a study by Ramakrishnan *et al.* (1974)1000 patients were investigated, the genetic nature of the disease was only apparent in 19 families who produced multiple cases. A comprehensive study by Marneros *et al.* (2004), has determined a link to chromosome 2q23 for a Japanese family and chromosome 7p11 for an African-American family, this study is thought to provide the first genetic evidence for keloid susceptibility loci.

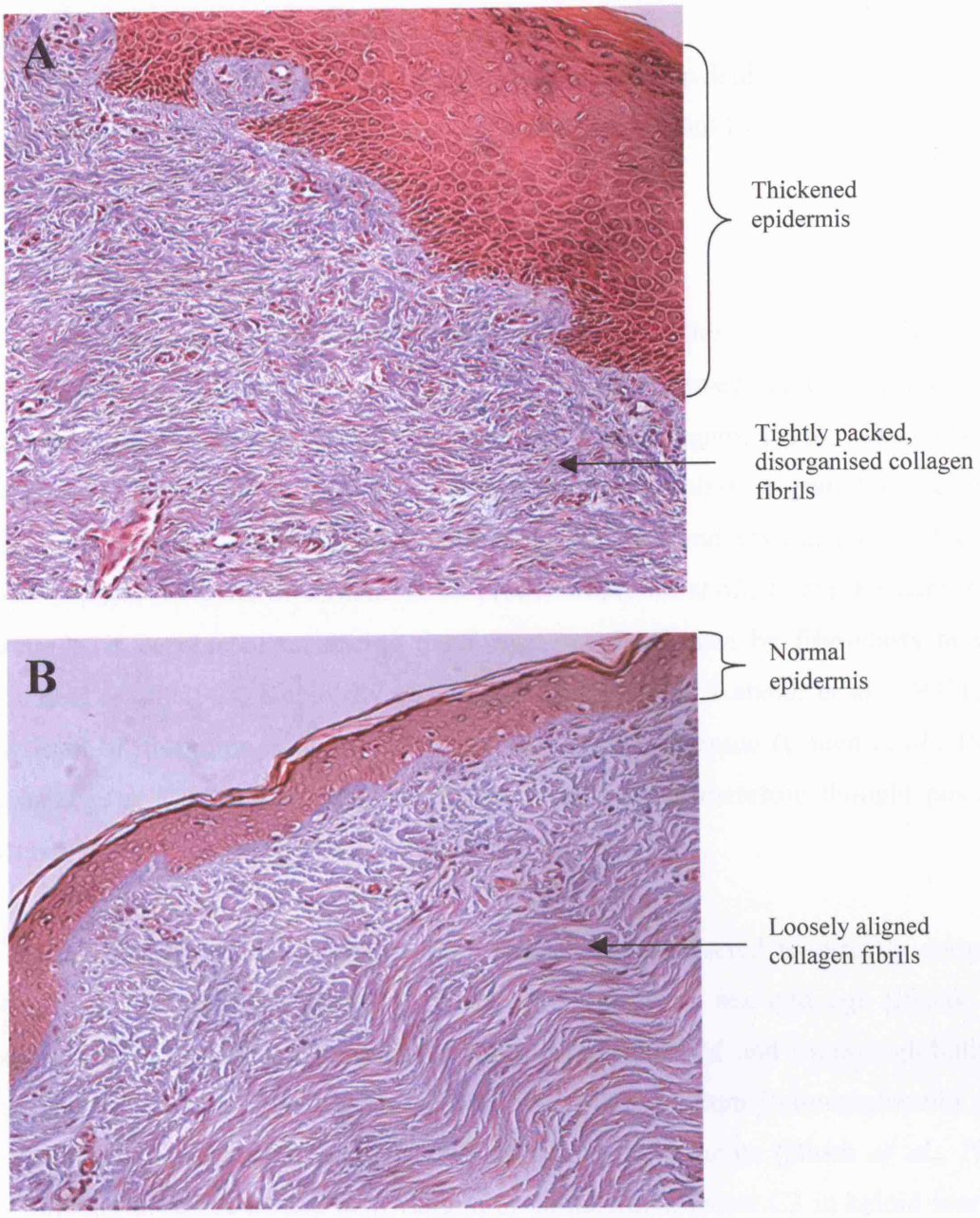

Figure 1.12 Masson's trichrome staining of keloid scar (A) and normal scar tissue (B) (x100 Mag) (Vigor C, unpublished data).

1.4.2.2 Immunological Involvement

The increased collagen synthesis and deposition, which is characteristic of keloids, may be related to an immune response initiated by wounding; various reviews and research articles provide strong evidence for this possibility as described below.

Blood Group Associations

Blood type A and cell membrane proteins such as human leukocyte antigen-B14, -B21, -BW16, BW35, -DR5, -DQW3 are linked with keloid scarring (Hunt, 1980; Castagnoli *et al.*, 1990; Castagnoli *et al.*, 1990).

Allergic Response

Keloid scar afflicted patients show a higher frequency of allergic symptoms compared to individuals with normal scars (Smith *et al.*, 1987). Increased numbers of mast cells have been implicated with keloid scars (Lee and Vijayasingam, 1995). Mast cells can be stimulated by immunoglobulin E to expel their cytoplasmic granules releasing: histamine, heparin, serotonin, acid hydrolase, chymase, and several growth factors, most of them involved in dermal matrix production (Ono *et al.*, 1995). Histamine for example, is capable of enhancing the formation of collagen by fibroblasts *in vivo* (Kikuchi *et al.*, 1995; Kupietzky and Levi-Schaffer, 1996; Kendall *et al.*, 1997) and the level of histamine is found to be increased in keloid tissue (Cohen *et al.*, 1972; Kischer *et al.*, 1982a). A relationship with mast cells is therefore thought possible (Atkins and Clark, 1987).

The serum levels of immunoglobulin E have been discovered to directly correlate with the incidence of excessive scar formation for race, sex and age (Placik and Lewis, 1992). Serum concentrations of immunoglobulin M and immunoglobulin G are also increased in keloid scarring patients, whereas serum immunoglobulin A is decreased compared with that of non-keloid forming patients (Bloch *et al.*, 1984) Conflicting evidence on the involvement of complement factor C3 in keloid scarring has been reported, however C4 is increased in non-keloidal forming patients (Bloch *et al.*, 1984). Patients with keloid scars also show anti-nuclear antibodies from different immunoglobulin classes against fibroblasts, epithelial and endothelial cells, this further suggests immunologic involvement in keloid scarring (Janssen de Limpens and Cormane, 1982).

Furthermore, keloid scar keratinocytes show expression of human leukocyte antigen class-2 and intercellular adhesion molecule-1 (ICAM-1), whereas normal scar keratinocytes do not. The increased expression of antigen-presenting properties of keratinocytes for T-lymphocytes recognition, suggests an immunologic involvement

by keratinocytes (Castagnoli *et al.*, 1994). Keloid scar keratinocyte behaviour is further discussed in section 1.4.2.10.

1.4.2.3 Clot Homeostasis

As detailed in section 1.3.1 immediately after wounding, platelet aggregation and activation of the clotting and complement cascades form a fibrin rich haemostatic clot. The breakdown of this clot is initiated by fibroblasts that exhibit a high plasminogen activator (PA) activity and a low plasminogen activator inhibitor-1 (PAI-1) activity, which in turn activates plasmin, responsible for clot lysis. Fibroblasts derived from keloid scars exhibit low levels of plasmin activator and high levels of inhibitor activity, which results in lower plasmin concentration and reduced degradation (Tuan *et al.*, 1996b). Inhibition of fibrin clot degradation can also be caused by TGF-β1 activity; where it down-regulates plasmin activator activity and up-regulates the activator inhibitor activity in normal dermal fibroblasts (Tuan *et al.*, 1996a). Indeed, TGF-β1 is found to be elevated in keloid scars (discussed further in section 1.4.2.9).

As previously mentioned in section 1.3.1, when platelets degranulate after aggregation they release and activate an array of growth factors along with fibronectin. This in turn recruits inflammatory cells, which activates granulation tissue formation (Tredget *et al.,* 1997). Excessive scar tissue contains increased amounts of fibronectin and granulation tissue. This has been suggested to indicate that there may be a disorder in platelet function in keloid scarring patients, leading to an exaggeration of wound healing processes, although direct evidence for this remains sparse.

1.4.2.4 Inflammatory Process

The evidence for the involvement of the inflammatory process in keloid scar pathology specifically is largely circumstantial. Nevertheless, it is clear that several aspects of this response are exaggerated and could therefore result in excessive scarring.

During inflammation, neutrophils and macrophages release several growth factors responsible for matrix production (Table 1.1). Chronic wounds such as large area trauma or infected wounds can exaggerate the inflammatory phase, which increases

the concentration of pro-fibrotic cytokines such as PGDF, TGF-β, and IGF-1, potentially leading to keloid formation in susceptible individuals.

As described previously in section 1.3.1, macrophages play a key role in the transition between the inflammatory and granulatory phases, whereby they are able to control and sustain the wound-healing events (Pierce, 1990). It has been hypothesised that in this manner, they can possibly initiate the formation of keloids by the release of fibroblast activating cytokines, such as TGF-β and PDGF (Wahl, 1985; Elias *et al.*, 1982). Indeed TGF-β levels (Hanasono *et al.*, 2003) and PDGF receptors (Haisa *et al.*, 1994) are elevated in keloid scar cells.

Macrophages also produce IL-1α and 1β, not only are these cytokines responsible for the stimulation of inflammatory cell adhesion and migration (Lowry, 1993), but they are also important in the degradation of the extracellular matrix. IL-1 and glucocorticoids stimulates the release of MMPs (Postlethwaite *et al.*, 1983, 1988), and increases collagenase activity in fibroblasts (McCauley *et al.*, 1992). IL-1 can also synergise with IFN-γ and TNF-α, released from inflammatory cells, to induce collagenase activity (Elias *et al.*, 1987; Meyer *et al.*, 1990). Decreased levels of IL-1 are found in patients with keloidal scars and correlates with the extent of their deformity (McCauley *et al.*, 1992). This suggests that a decrease in wound levels of IL-1 may result in extracellular matrix accumulation and scar formation (Niessen *et al.*, 1999).

Cells within excessive scar tissue also produce increased levels of dermatan- and chondroitin sulphates, fibronectin and collagen, which are enhanced by IL-4 (Postlethwaite *et al.*, 1992; Wegrowski *et al.*, 1995) leading to the suggestion that IL-4 may be involved in keloid scarring. Although no reports giving direct evidence of the latter have been published, there are studies describing abnormal levels of IL-6 in keloid scar fibroblasts specifically (Xue *et al.*, 2000) and IL-13 and -15 in pathological scars (Castagnoli *et al.*, 1999; Oriente *et al.*, 2000). Interleukins are a variety of substances produced mainly by leukocytes and are of the larger class of T-cell products, *lymphokines,* frequently considered as chemokines. Broadly, interleukins mediate and control the immunologic and inflammatory response.

IFN-α and -γ, which act to down-regulate collagen synthesis are found to be deficient in keloid scars (Elias *et al.*, 1987; Berman and Duncan, 1989; Berman and Duncan, 1989; Larrabee *et al.*, 1990; McCauley *et* al., 1992). Furthermore, IFN-α, -β and -γ all inhibit the proliferation of rapidly dividing fibroblasts and their collagen synthesis (Elias *et al.*, 1987b; Harrop *et al.*, 1995). IFN-γ is already used successfully in the treatment of excessive scar formation (Larrabee *et al.*, 1990; Granstein *et al.*, 1990). Reduced IFN-α, IFN-γ and TNF-α concentrations in keloid scar tissue suggests that an altered inflammatory response, elicited by keloid scarring individuals may play a role in the potentially aberrant synthesis and breakdown of collagen by Keloid scar-derived fibroblasts (McCauley *et al.*, 1992; Castagnoli *et al.*, 1993; Peruccio *et al.*, 1994).

1.4.2.5 Angiogenesis

Keloid scars contain an excessive number of microvessels when compared with normal scars; possibly suggesting an overactive environment which demands increased oxygen levels or an increase in VEGF, stimulating excessive angiogenesis (Steinbrech *et al.*, 1999). Collagen concentrates between the lateral branches of the new microvessels in excessive scar tissue, this deposition results in collagen nodules of various shapes and sizes (Kischer *et al.*, 1990). The microvessels of keloid scars start to become occluded by excess endothelial cells during the late granulation tissue remodelling stage (Kischer *et al.*, 1982b; Lawrence, 1987). Blood vessel occlusion may be the cause of reduced tissue oxygen concentration in excessive scar tissue (Knighton *et al.*, 1981); although, it may also be due to either high tissue metabolic rates or reduced diffusion of oxygen to the wound space (Sloan *et al.*, 1978). Nonetheless, the resulting hypoxia is potentially able to stimulate angiogenesis by activating factors released from macrophages (Knighton *et al.*, 1983), including PDGF and bFGF (Kuwabara *et al.*, 1995) and inciting fibroblasts to proliferate and produce collagen; possibly accounting for the bulk of excessive scar tissue (Kischer *et al.*, 1982a, 1982b). During scar maturation, the microvessels slowly but progressively degenerate and are absorbed; this correlates with the formation and enlargement of collagen nodules (Kischer, 1993).

It has also been suggested that a cell-type distinct from that of the fibroblast is involved in the induction of keloid scarring. Namely the pericytes; pericytes are smooth muscle cells that surround blood vessels; they may however serve as dermal fibroblasts and produce collagen. Pericytes also express α-SMA, characteristic of myofibroblasts (Sappino and Schurch, 1990). Hypoxic conditions can cause continued proliferation of pericytes, which are thought to contribute towards the occlusion of the blood vessel lumen (Kischer *et al.*, 1982b; Kischer and Hendrix, 1983). Currently, there is no reliable marker to distinguish the origin of wound myofibroblasts, whether they are derived from pericytes or fibroblasts, or possibly progenitor stem cells.

1.4.2.6 Extracellular Matrix Homeostasis

The formation of the extracellular matrix is through the synthesis of collagens, fibronectin and proteoglycans, all carried out by fibroblasts as considered previously in section 1.3.3. The degradation of the extracellular matrix is carried out by collagenases, and other proteases, released by mast cells, macrophages, endothelial cells and fibroblasts. An alteration in the delicate balance that must exist between these two processes could potentially lead to the formation of keloid scars. As fibroblasts play such an important role in extracellular matrix homeostasis they are thought to play a key role in keloid scar development. The density and activity of fibroblasts is increased in excessive scar tissue (Matsuoka *et al.*, 1988; Nakaoka *et al.*, 1995; Calderon *et al.*, 1996), but their proliferation is relatively normal (Oku *et al.*, 1987). Studies have found that keloid scar cells express normal growth parameters, i.e., mean population doubling time, density, cell volume and karyotype (Russell and Witt, 1976). Keloid scar fibroblasts however, produce an abnormal amount of extracellular matrix proteins; collagen, fibronectin, elastin and proteoglycans compared with dermal fibroblasts and normal scar fibroblasts (Kurkinen *et al.*, 1980; Oliver *et al.*, 1992). For instance, in normal scars fibronectin disappears within a few days after wound closure, but in keloid scars the fibronectin activity continues at a high level for months or years, suggesting prolonged activation of these cells (Kischer and Hendrix, 1983). Keloid scars also express increased prolyl-4-hydroxylase activity, collagen mRNA expression and elevated gene transcription of α1(I)-procollagen (Abergel *et al.*, 1985; Lee *et al.*, 1991; Ghahary *et al.*, 1996).

Keloid scar fibroblasts show aberrant responses after stimulation with metabolic stimulators, compared with normal dermal fibroblasts (Babu *et al.*, 1992; Russell *et al.*, 1995; Tuan and Nichter, 1998). An example of this is seen with the effect of TGF-β1 on fibronectin synthesis; where although the production of fibronectin is accelerated in both normal dermis and keloid scar-derived fibroblasts treated with TGF-β1, it occurs much more rapidly in keloid scar fibroblasts (Babu *et al.*, 1992), implicating an increased sensitivity of keloid fibroblasts to TGF-β1. This implies that keloid scar cells have an altered response to their growth environment with regards to the synthesis of extracellular matrix and soluble molecules. Fibroblasts from keloid scars may additionally have a defective system of down-regulating their extracellular matrix metabolism (Sato *et al.*, 1998). Keloids have been found to have an abnormally increased production of collagen in comparison to normal dermis and normal skin (Diegelmann *et al.*, 1979).

The characterisation of keloid scars with excessive deposition of collagen and other extracellular matrix proteins could possibly result from insufficient protein degradation. Keloid scar cell cultures have been reported to exhibit reduced degradation of newly synthesised collagen polypeptides, suggesting reduced collagenase activity (Abergel *et al.*, 1985; Ghahary *et al.*, 1996). Collagenase inhibitors such as α2-macroglobulin and α1-anti-trypsin are found by some groups to be accumulated in keloid scars (Diegelmann *et al.*, 1977; Ueyama *et al.*, 1992). However, there is conflicting evidence of this (Milsom and Craig, 1973; Cohen *et al.*, 1975; Diegelmann *et al.*, 1979; McCoy and Cohen, 1982; Abergel *et al.*, 1985; Berman and Bieley, 1995). Neely *et al* (1999), found increased levels of MMP-2 in keloid scar tissue in comparison to normal dermal tissue, and Fujiwara *et al* (2005), found MMP-2 and MMP-1 increased by 6-fold and 2.4-fold, respectively in keloid scar fibroblasts in comparison to normal dermal fibroblasts. The increased production of MMPs may have an important role in the high migratory activity of keloid scar fibroblasts (Fujiwara *et al.*, 2005).

1.4.2.7 Collagen

As previously mentioned in section 1.3.3.1, during wound repair, collagen type III first appears in the wound site at around day 2-3 followed by collagen type I at around

day 6-7, whereas type V increases in parallel with tissue vascularity (Diegelmann *et al.*, 1975; Betz *et al.*, 1992). Over the wound repair period the content of the wound changes from consisting of mainly type III collagen (60% after wounding reducing to 28% in the mature scar) to that of collagen type I (Holslev-Petersen *et al.*, 1988). In keloid scars however, the relative amount of collagen type III remains increased compared with normal scar and normal dermis, which is shown by the ratio of collagen III to collagen I (Weber *et al.*, 1978b; Uitto *et al.*, 1985; Di Cesare *et al.*, 1990; Lee *et al.*, 1991; Friedman *et al.*, 1993). The ratio of type III/I procollagen mRNA expression in keloids is about 0.61, whereas in normal skin the ratio is 0.17 (Lee *et al.*, 1991).

During normal wound repair, collagen synthesis reaches its peak at about 6 months after injury and then starts to decline to a normal turnover 2-3 years after wounding (Craig *et al.*, 1975; Abergel *et al.*, 1985; Muir, 1990). In keloid scars, collagen synthesis can remain high for several years (Diegelmann *et al.*, 1979).

The synthesised collagen fibrils in normal scars are smaller, more regular and show a higher inter-fibrillar distance compared with keloid scars. The irregular orientation of the collagen fibrils in keloid scars could be responsible for the fact that they fail to participate in scar contractures (Ehrlich *et al.*, 1994); although this may simply be due to increased collagen type III in keloid scars. The collagen fibres of keloid scars are more acid soluble (Bazin *et al.*, 1973; Bailey *et al.*, 1975; Bailey *et al.*, 1975b), show a decreased amount of lysyl oxidase-dependent complex cross-linking, and fail to mature compared with normal collagen (Knapp *et al.*, 1977; Di Cesare *et al.*, 1990). This again implies a possible fault with the enzymes involved in organising and remodelling the collagen matrix, preventing cells from contracting the wound matrix and consequently prolonging the second stage (fibro-proliferative phase) of wound healing.

An increase in biglycan, collagen type I and hyaluronic acid is found in keloid scar tissue (Hunzelmann *et al.*, 1996; Alaish *et al.*, 1995). The organisation and degradation of collagen fibres is affected by the concentrations of these proteoglycans. Hyaluronic acid, decorin and biglycan can bind to collagen fibres and influence their 3-dimensional arrangement (Linares and Larsen, 1978; Poole *et al.*,

1982; Hunzelmann *et al.*, 1996). Although decorin has not been implicated in keloid scarring, both decorin and biglycan can interact with collagen type I fibres, which can influence their fibrillogenesis (Fleischmajer *et al.*, 1991; Schonherr *et al.*, 1995). They can also inhibit fibroblast adhesion to matrix components and can bind TGF-β, possibly regulating its behaviour (Yamaguchi *et al.*, 1990; Bidanset *et al.*, 1992; Bidanset *et al.*, 1992). The coordinated role of collagen and proteoglycans in excessive scar tissue formation is supported by an up-regulation of both these protein types in active keloid scars.

1.4.2.8 Matrix Remodelling

After the deposition of the early extracellular matrix, the cells within the matrix begin to remodel the collagen framework and proteoglycan filler substance to obtain the scar's ultimate strength (Niessen *et al.*, 1999). Hyaluronic acid is part of the extracellular matrix filler substance and a major component of early granulation tissue. Hyaluronan facilitates the movement and division of cells within the extracellular matrix (Docherty *et al.*, 1989; Bertolami *et al.*, 1992; Meyer and Stern, 1994). The concentration of hyaluronic acid normally increases initially after wounding, decreasing from days 5-10, and then remains fairly constant while the sulphated glycosaminoglycans, chondroitin-4-sulphate and dermatan-sulphate, increase concomitantly (Burd *et al.*, 1991; Clark, 1996). In conditions of excessive tissue fibrosis including keloids however, hyaluronic acid and the proteoglycans remain at supra-normal levels (Alaish *et al.*, 1995).

The latter observation follows earlier suggestions with regards to an abnormality in the production of extracellular matrix components by keloid fibroblasts. These cells are seemingly over stimulated by means of these components leading to highly activated cells which are not being cleared by apoptosis, but continue to proliferate and remain as active factories of wound mediators. Furthermore, hyaluronic acid is concentrated in the thickened granular and spinous layers of the epidermis, implying an over activity of epidermal keratinocytes in keloid scar (Bertheim and Hellstrom, 1994).

Hyaluronic acid is known to form a pericellular coat on the surface of cells; this is suggested to be mediated by cell surface receptors (Yoneda *et al.*, 1990). It is

speculated that hyaluronic acid could play a role this way in maintaining TGF-β1 around the cell's microenvironment (Mast *et al.*, 1993), while potentiating the bioactivities of TGF-β1. The interaction of TGF-β1 with hyaluronic acid may result in a stable complex that resists degradation by enzymes and, thereby, maintains the bioactivity of TGF-β1 as a collagen production stimulator (Shah *et al.*, 1992; Alaish *et al.*, 1995). If the hyaluronic acid pericellular boundary not only localises cytokines such as TGF-β1, but also stabilises the plasma inhibitor-collagenase complexes, then collagen production would be increased and degradation would be inhibited, resulting in excessive scar tissue (Alaish *et al.*, 1995).

1.4.2.9 Growth Factors

TGF-β and PDGF

TGF-β is one of the most well studied growth factors and appears to be an important factor in excessive scar formation (Leask and Abraham, 2004). The first step in a fibrotic reaction is the expression of the TGF-β1 gene by inflammatory and endothelial cells. On its release and activation, TGF-β promotes fibroblast proliferation and the synthesis of extracellular matrix components such as elastin, fibronectin, and collagen types I and III (Peltonen *et al.*, 1991). Keloid scar-derived fibroblasts are more sensitive to TGF-β stimulation (Tredget, 1994). The mitogenic response of keloid scar fibroblasts requires a lower concentration of the growth factor compared with normal dermal fibroblasts (Russell *et al.*, 1988). This sensitivity could be related to differences in receptor number, affinity or specific receptor type on the cells surface. In comparison to dermal fibroblasts, keloid scar fibroblasts are reported to have higher: autocrine production of TGF-β1 (Lee *et al.*, 1999; Hanasono *et al.*, 2003; Chen *et al.*, 2004), receptor number and Smad phosphorylation (Chin *et al.*, 2001), along with increased collagen synthesis (Younai *et al.*, 1994), fibronectin production (Babu *et al.*,1992; Chen *et al.*, 2003b) and DNA synthesis (Bettinger *et al.*, 1996) in response to exogenous TGF-β1. This reduced growth factor requirement may explain the exuberant growth of keloids into tumour-like masses (Russel *et al.*, 1988).

Amongst TGF-β's plethora of effects, it has been found to reduce the collagenase-mediated degradation of the wound matrix (Overall *et al.*, 1989), although research in this area is confusing as Sato *et al* (1998), who also found TGF-β1 to down-regulate

the production of MMP-1 (collagenase-1), found that TGF-β1 can in fact increase MMP-2 (gelatinase-A) production.

TGF-β1 stimulation of extracellular matrix deposition has been determined to occur indirectly by the induction of PDGF (Leof *et al.*, 1986). PDGF has been shown to be responsible for an acceleration of granulation tissue formation (Lepisto *et al.*, 1992) and for the stimulation of collagen production during the later stages of wound healing (Pierce *et al.*, 1992; Lawrence and Diegelmann, 1994). Keloid scar fibroblasts show an increased mitogenic and chemotactic response to PDGF compared to normal dermal fibroblasts; suggested to be mediated by elevated levels of PDGF α-receptors, which are 4-5 times higher than those in normal dermal fibroblasts (Haisa *et al.*, 1994). In contrast to this finding however, Kikuchi *et al* (1995) reported no difference in mitogenic response when comparing keloid scar-derived fibroblasts to normal dermal fibroblasts.

Further proof of the importance of TGF-β in the development of excessive scar tissue has been shown by the use of neutralizing antibodies to TGF-β1 and -β2, as well as the use of Smad 7 (inhibitor of TGF-β1 signalling pathway via Smads), and antisense oligonucleotides (Shah *et al.*, 1992, 1995; Border and Noble, 1998; Nakao *et al.*, 1999). Shah *et al* (1992) found that with the addition of neutralising antibodies to TGF-β1, wounds healed with reduced scar formation, they also found the wounds to contain fewer macrophages and blood vessels, with a decreased content of collagen and fibronectin, but identical tensile strength and more normal dermal architecture than non-treated control wounds. In addition, Nakao *et al* (1999) found that the addition of an adenovirus carrying Smad-7 to mice with bleomycin-induced lung fibrosis, caused decreased type I pro-collagen mRNA, reduced hydroxyproline content and no morphological fibrotic responses in the lungs compared to control mice.

The local administration of exogenous TGF-β3 in contrast to TGF-β1 and -β2 has been demonstrated to reduce scarring in adult animal models (Shah *et al.*, 1995). Ferguson and O'Kane (2004), found embryonic animal wounds that heal without scar formation to express low levels of TGF-β1, TGF-β2, PDGF and high levels of TGF-

β3. This work further demonstrates the roles of TGF-β1, -β2 and -β3 in fibrosis. It has been postulated that an abnormality in the cellular response of keloid to TGF-β1 may lead to an aberration in TGF-β1-mediated events, such as increased synthesis of extracellular matrix components and reduced matrix breakdown. The latter of these is essential to conclude the proliferative stage of wound healing and encourage wound contraction and eventually cell quiescence. The role of TGF-β1 in tissue fibrosis is discussed in further detail in section 6.1.

EGF and bFGF

Investigations of the effects of EGF and bFGF on keloid scar cells are relatively few and inconsistent; where EGF or bFGF stimulation has been reported to exhibit no effect (Haisa *et al.*, 1994; Hanasono *et al.*, 2003), in contrast to reports of a dose-dependent enhancement of granulation tissue formation by bFGF stimulation (Goretsky *et al.*, 1996), and a down-regulation of collagen production by EGF stimulation (Harper, 1989; Tan and Peltonen, 1991; Tan *et al.*, 1993) in comparison to normal dermal fibroblasts. The variations in results however, may be due to different experimental designs.

IGF-I

During wound repair IGF-I acts to increase the expression of types I and III procollagen (Ghahary *et al.*, 1995) and reduces MMP-1 mRNA activity (Ghahary *et al.*, 1996). This polypeptide also acts as a cell survival and differentiation factor. Increased levels of IGF-I have been reported after tissue injury and in post-burn hypertrophic scars (Ghahary *et al.*, 1995). In keloid scar fibroblasts the IGF-1 receptor is over-expressed (Yoshimoto *et al.*, 1999; Ohtsuru *et al.*, 2000; Ishihara *et al.*, 2000), suggesting another possible mechanism for the aberrant collagen expression levels in keloid scars. In addition the invasive potential of keloid fibroblasts over-expressing the IGF-1 receptor is increased (Yoshimoto *et al.*, 1999; Ohtsuru *et al.*, 2000).

1.4.2.10 Keratinocytes and Altered Growth Factor Expression

As well as fibroblasts, keratinocytes are an important source of growth factors, most of which are involved in the inflammatory processes in the wound area (McKay and Leigh, 1991; Ansel *et al.*, 1993; Bennett and Schultz, 1993). Some of these growth

factors such as: TGF-β, PDGF, bFGF, IL-1, and TNF-α are known to play an important role in granulation tissue formation and in scar remodelling (Mustoe *et al.*, 1991; Pierce *et al.*, 1991; Quaglino *et al.*, 1991; Kessler *et al.*, 1992; Mellin *et al.*, 1992; Pierce *et al.*, 1992; Lawrence and Diegelmann, 1994; Shah *et al.*, 1995). Recent research co-culturing normal dermal fibroblasts with keloid scar-derived keratinocytes have identified keloid scar keratinocytes as able to promote the proliferation, collagen production and inhibit the apoptosis (induced through serum deprivation) of normal dermal fibroblasts (Lim *et al.*, 2001; Phan *et al.*, 2002; Lim *et al.*, 2002; Funayama *et al.*, 2003). This implies that keloid scar-derived keratinocytes may potentially be involved the deregulation of growth factors detected in keloid scars.

1.4.2.11 Activated Fibroblasts/Myofibroblasts

As well as scar-tissue formation and remodelling, the wound reduces its surface area by contraction; fibroblasts are of prime importance to this process. At around day 9 of wound healing, fibroblasts develop into their activated secretory phenotype and eventually myofibroblasts (Darby *et al.*, 1990; Clark, 1996). When contraction stops and the wound is fully epithelialised, the myofibroblasts alongside other redundant cells disappear via apoptosis (Gabbiani, 1992; Desmouliere, 1995).

Findings on the presence of myofibroblasts in keloids have been contradictory; some research groups describe keloid scar tissue as having few collagen nodules and although activated fibroblasts are present (judged by the presence of rough endoplasmic reticulum), they report a general absence of myofibroblasts (Matsuoka *et al.,* 1988; Ehrlich *et al.*, 1994). In contrast, others describe a persistence of abnormally thick, misaligned collagen fibres with collagen nodules and the increased expression of α-SMA and fibronectin; myofibroblasts being the predominant cell type in keloid scars (James *et al.*, 1980; Kischer *et al.*, 1982a; Lee and Vijayasungam, 1995; Santucci *et al.*, 2001). These conflicting findings may be due to the differences in age of the keloid scars studied, but alternatively could indicate an altered state of activity. The type, age and area of a keloid lesion correlate with the different amounts of immune cells present in the scar tissue, and extracellular matrix content and organisation (Santucci *et al.*, 2001). Irrespective of whether the keloid scar fibroblasts are the fully mature myofibroblast phenotype or just highly activated, secretory fibroblasts, the prolonged presence of these cells might explain the excessive nature of

these scars. In addition, the persistence of the myofibroblast phenotype in pathological scars may be the cause of excessive scar contracture, which is seen more extensively in hypertrophic scars (Brody *et al.*, 1981; Ehrlich *et al.*, 1994; Tsai *et al.*, 1995). In culture, both hypertrophic scar and keloid fibroblasts express similar amounts of α-SMA, suggesting that local factors influence their expression (Ehrlich *et al.*, 1994), for example the composition of the matrix itself or paracrine secretion from leukocytes and keratinocytes *in vivo*. An important local factor seems to be TGF-β1, which induces the expression of α-SMA in cultured fibroblasts and has also proved to induce collagen and fibrin-matrix contraction (Desmouliere *et al.*, 1993; Younai *et al.*, 1996; Lijnen *et al.*, 2003). It also stimulates the autocrine production of PDGF involved in fibroblast activation and wound contraction (Younai *et al.*, 1996). As previously mentioned, keloid scar-derived fibroblasts are more sensitive to both TGF-β and PDGF, which may explain the persistent presence of the activated fibroblast phenotype in this tissue.

1.5 Rationale for the Research Direction of this Thesis

Although all of the factors discussed above may contribute to this disease, the fundamental fault in keloid scars would appear to be with the balance that should exist between proliferation and apoptosis. As formerly discussed, kinetic studies have shown that keloid fibroblasts do not differ significantly from that of normal dermal fibroblasts when grown in culture in the presence of fetal calf serum (Russell and Witt, 1976; Diegelmann *et al.*, 1979). However, keloid scar fibroblasts do have a reduced growth factor requirement (Russell *et al.*, 1988), and immature keloid scar tissue exhibits vast numbers of active fibroblasts/myofibroblasts along with an increased expression of thickened misaligned collagen fibrils (James *et al.*, 1980; Kischer *et al.*, 1982a; Lee and Vijayasungam, 1995; Santucci *et al.*, 2001). It is possible therefore, that keloid scars occur due to a prolonged existence of activated fibroblasts, leading to the continued production of extracellular matrix components, growth factors and cytokines. This could theoretically be caused by unremitting induction of these cells or their failure to undergo apoptosis (Appleton *et al.*, 1996). Absence of or even a delay in apoptosis of keloid scar fibroblasts and continued production of vast amounts of extracellular matrix would potentially result in a very active, cellular, collagenous scar.

Although cells can potentially disappear from a wound by either necrosis, or emigration, apoptosis appears to be the main cause of decreasing cellularity during wound healing (Desmouliere *et al.,* 1995). Apoptosis is the final critical phase of wound healing, involved in clearing the wound site of the redundant fibroblasts no longer required. This study concentrates on the induction of apoptosis to aid wound clearance and re-establish tissue quiescence. The process of apoptosis (programmed cell death) is very complex and dynamic, involving many overlapping inducible signalling pathways, which coordinate with each other, along with many inhibitory pathways to prevent uncontrolled escalation of cell death. A thorough understanding of these pathways is needed before investigating and dissecting specific signalling pathways, for the analysis of wound-healing induced apoptosis.

1.6 Apoptosis

A number of reviews have covered the events that occur during apoptosis (Wyllie *et al.*, 1980; Duke *et al* 1983; Arends *et al.,* 1990; Cohen, 1997; Gross *et al.*, 1999; Morita and Tilly, 1999; Slee *et al.*, 1999; Fadeel and Orrenius, 2005) a summary of these is considered below, additional data is cited appropriately.

Apoptosis is the controlled induction of cell death of unwanted cells which, unlike necrosis occurs in a contained fashion so that no collateral damage is caused. Apoptosis is inducible via extrinsic (outside stimulus to the cell) or instrinsic (mitochondrial) control mechanisms. Primarily apoptosis is thought to involve the expression of an endogenous endonuclease that cleaves DNA. Typical morphological changes include: condensation and fragmentation of the nucleus and modifications of cytoplasmic organelles (Searle *et al.,* 1982). Whereas during necrosis, the intracellular constituents, including proteolytic enzymes are released into the extracellular environment where they cause considerable local damage and result in inflammation; in contrast cells that undergo apoptosis do not. Instead, the cell membrane remains intact and apoptotic cells are removed thorough phagocytosis either by macrophages or neighbouring cells.

There are four main stages leading to apoptosis (Figure 1.13): a harmful stimulus interacts with the cell, early signalling molecules are activated, a regulatory

mechanism evaluates the strength of the apoptosis signal compared with anti-apoptotic signals, and if the balance is towards apoptosis, specific executioner proteins are activated which are responsible for the organised destruction of the cell.

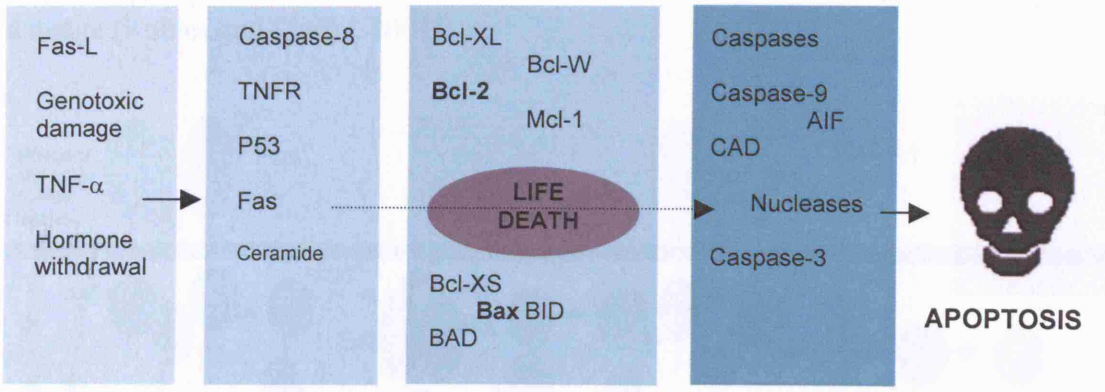

Figure 1.13 Main stages of apoptosis induction (adapted from Wyllie *et al.*, 1980; Morita and Tilly, 1999).

1.6.1 Extrinsic Control Mechanisms

In normal physiological cell turnover the pro-apoptotic stimuli can be represented by growth factor/cytokine deprivation, or death factors, such a Fas ligand (FasL) or TNF-α (Nagata, 1994). These death factor proteins belong to the same transmembrane protein subfamily, and can be generated into soluble forms by metalloproteinase-mediated cleavage (Krammer, 1999; Schmitz *et al.*, 2000). Fas and TNF death receptors are characterised by unique intracellular death domains (DD), which are crucial for death ligand-induced apoptosis (Figure 1.14) (Huang *et al.*, 1996). The binding of death ligand to its receptor leads to trimerisation of the receptors (Nagata, 1994). The subsequent association of three death domains leads to the formation of a death-inducing signalling complex (DISC), which leads to the activation of pro-caspase-8. The message to induce apoptosis can be modulated directly at the death receptor level and at a transcriptional level. As well as this, a class of proteins named FLIPs (FLICE/Caspase-8 like inhibitory proteins) (Thome *et al.*, 1997) can block apoptosis by directly interacting with the death receptor pathway (Figure 1.14) (Hu *et al.*, 1997; Bertin *et al.*, 1997).

In addition to physiological control mechanisms of apoptosis, a variety of pathological insults can trigger apoptosis. Factors that are capable of causing DNA damage, such as radiation, cytostatic drugs or genotoxic compounds, can also induce

apoptosis (Bratton and Cohen, 2001; Wahl and Carr, 2001). The activation and stabilisation of p53 occurs due to a variety of signals including; cytokine deprivation (Canman *et al.*, 1995), hypoxia and heat-shock (Graeber *et al.,* 1994) and DNA damage (Robles and Harris, 2001).

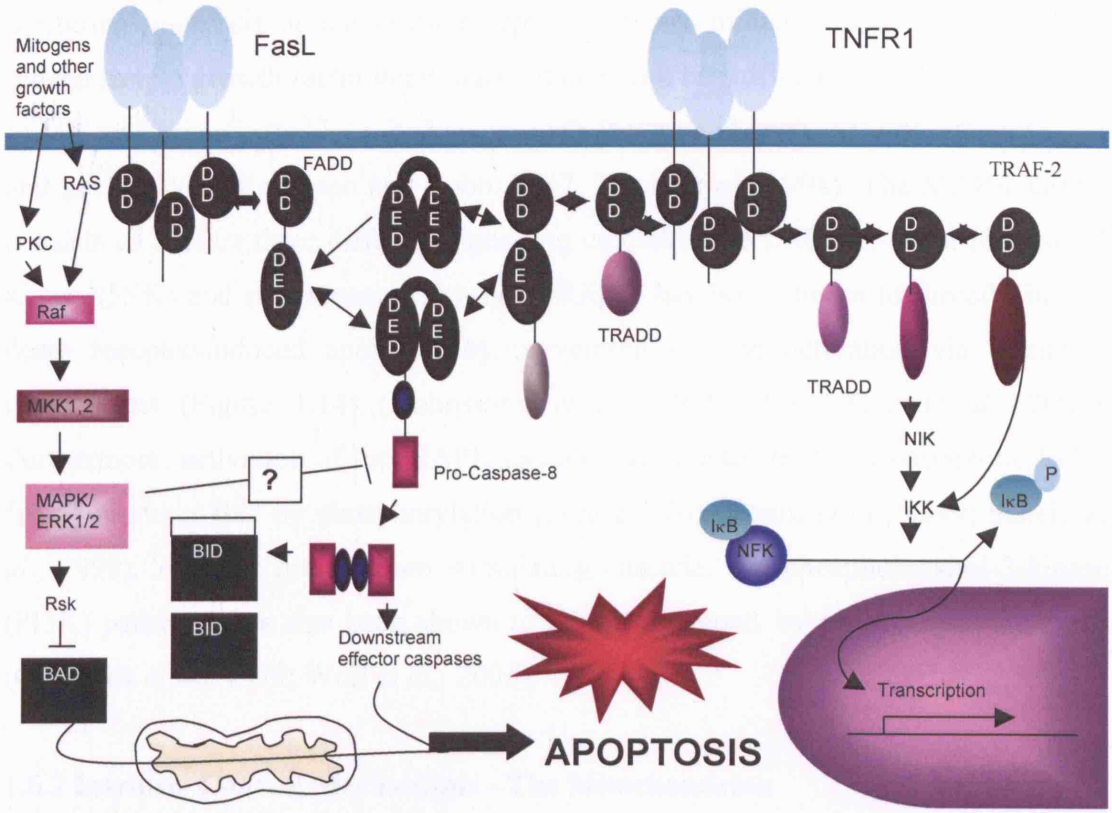

Figure 1.14 Regulation of death receptor signalling through extrinsic mechanisms. The activation of a Fas trimer by a FAS ligand (FASL) recruits an adaptor protein called FADD (Fas adaptor death domain) through interaction with their respective death domains. FADD functions as a bridge between Fas and downstream signal transduction. The Tumour necrosis factor receptor-1 (TNFR1) mediated pathway utilises TRADD (TNF adaptor death domain) protein in recruitment of FADD. The binding of pro-caspase-8 to the FAS/FADD or alternatively the TNFR1/TRADD/FADD complex activates auto-processing of the pro-enzyme to its active form. Activated caspase-8 is able to induce apoptosis through a mitochondrial pathway by cleaving BID, or directly by activating downstream effector caspases. Mitogens and growth factors can directly inhibit the activation of caspase-8 through an unknown pathway. Additionally the activation of mitogen-activated protein kinase/extracellular signalling-regulated kinase (MAPK/ERK) pathway can lead to phosphorylation, e.g. inactivation of BAD. TNFR1 also utilises an anti-apoptotic signalling pathway. TNF receptor associated factor (TRAF2) can bind to TNFR1/TRADD complexes and activate nuclear kinase protein (NIK) and consequently the IKappaB kinase (IKK) complex, a pathway that leads to phosphorylation of IκB and activation of transcription factor NFκB. (Adapted from Wyllie *et al.*, 1980; Morita and Tilly, 1999).

Depending on the damage and the cell type, p53 will either cause arrest in the cell cycle or activate the apoptotic self-destruction sequence (Vousden, 2002). p53 can induce the transcription of a number of pro-apoptotic genes such as bax and Fas (Miyashita and Reed, 1995; Owen-Shaub *et al.*, 1995).

Inhibiting apoptosis at the death receptor level are mitogens and growth factors. Indeed simple growth factor deprivation of cells can be sufficient to induce apoptosis. MAPK can be activated by mitogens, growth factors and environmental stress (Seger and Krebs, 1995; Robinson and Cobb, 1997; Lewis *et al.,* 1998). The MAPK family consists of at least three different signalling cascades: the ERK1/2, c-Jun-N-terminal kinase (JNK) and p38 kinase pathways. ERK1/2 has been shown to directly inhibit death receptor-induced apoptosis by preventing caspase activation via unknown mechanisms (Figure 1.14) (Holmstrom *et al.*, 1998, 1999; Tran *et al.*, 2001). Furthermore, activation of the MAPK cascade can inactivate the pro-apoptotic Bcl-2 family member Bad by phosphorylation (Figure 1.14) (Bonni *et al.*, 1999; Scheid *et al.*, 1999). Another proliferation stimulating cascade, the phosphoinositol-3-kinase (PI3K) pathway, has also been shown to inhibit apoptosis by phosphorylating BAD (Craddock *et al.*, 1999; Wolf *et al.,* 2001).

1.6.2 Intrinsic Control Mechanisms - The Mitochondrion

The mitochondrion is not just the powerhouse of the cell; it is also involved in the intrinsic apoptosis-regulating pathway. The mitochondrion acts to either enhance or silence apoptotic signals, this mitochondrion mechanism is regulated by members of the Bcl-2 family (Table 1.3).

On receiving apoptotic signals the pro-apoptotic Bax protein is relocated from the cytoplasm of the cell to the mitochondrion. Anti-apoptotic members of the Bcl-2 family, such as Bcl-2 itself and Bcl-X_L can block the pro-apoptotic effects of Bax by binding it and forming heterodimers. However, other pro-apoptotic Bcl-2 proteins, e.g. Bad and Bid, can interact with Bcl-2 and Bcl-X_L and prevent their anti-apoptotic function. Eventually, the relationship between pro-apoptotic and anti-apoptotic factors determines the susceptibility of the cell to apoptosis.

If there are more pro-apoptotic factors, the mitochondrion subsequently loses its membrane potential and a number of apoptosis-promoting molecules, such as cytochrome c and apoptosis-inducing factor (AIF) are released into the cytoplasm (Figure 1.15).

Table 1.3. Anti- and pro-apoptotic Bcl-2 family members

Anti-Apoptotic	Pro-Apoptotic
Bcl-2	Bax
Bcl-XL	Bak
Bcl-w	Bad
Mcl-1	Bcl-XS
A1	Bik/Nbk
	Bim
	Hrk
	Bid

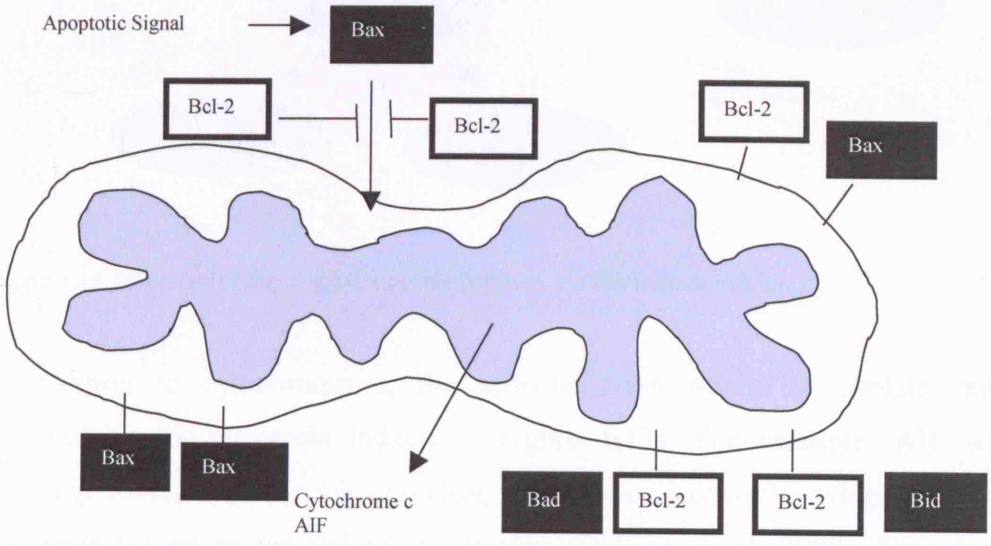

Figure 1.15 Representation of the interactions between anti-apoptotic (white) and pro-apoptotic (black) members of the Bcl-2 family at the outer mitochondrial membrane.

1.6.3 The Apoptosome

On release from the mitochondria cytochrome c associates with apoptosis protease-inducing factor-1 (Apaf-1) and procaspase-9 (Zou *et al.* 1997). Together these three factors create a holoenzyme termed, 'the apoptosome', which is a key connection between mitochondria and caspase activation (Figure 1.16). The apoptosome proceeds to activate caspase-3 and other death effector caspases that are required for the final stages of apoptotic cell death (Adrain & Martin 2001).

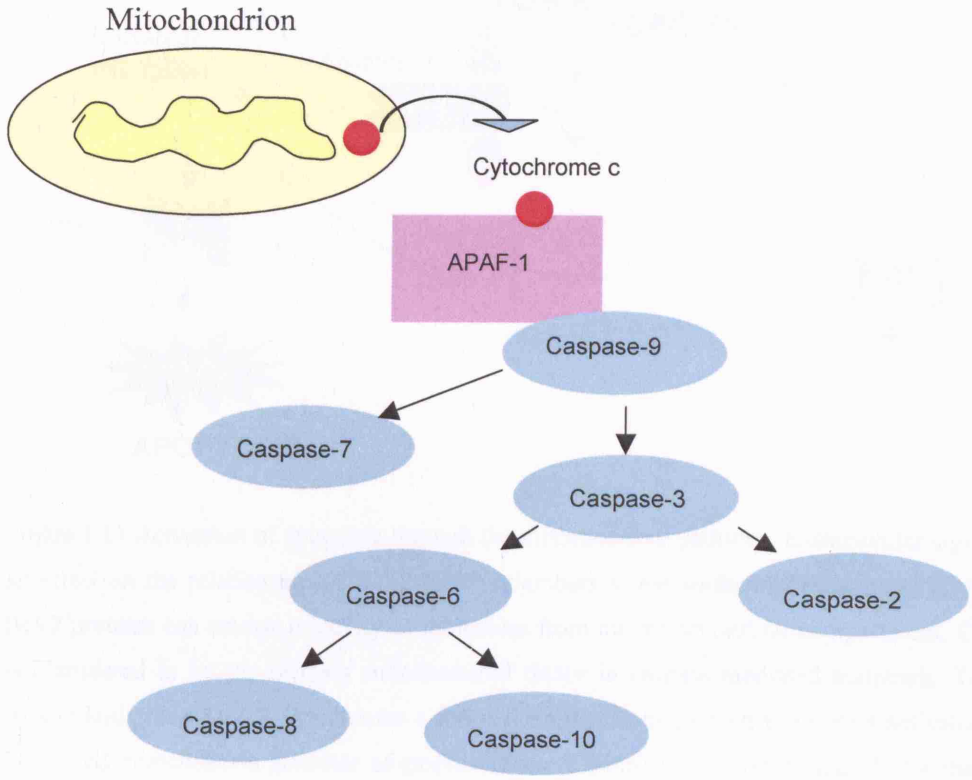

Figure 1.16 Induction of the caspase cascade pathway via the mitochondrion.

In addition to cytochrome c, the mitochondrion seems to contain redundant mechanisms for apoptosis induction (Figure 1.17). For example, AIF which is normally contained in the mitochondria, when dissociated to the cytoplasm, it induces apoptosis via an as yet unknown pathway (Daugas *et al.* 2000). However, AIF-induced apoptosis appears to be independent of caspase activation and it cannot be inhibited by Bcl-2 overexpression (Susin *et al.* 1999, Zamzami & Kroemer 1999).

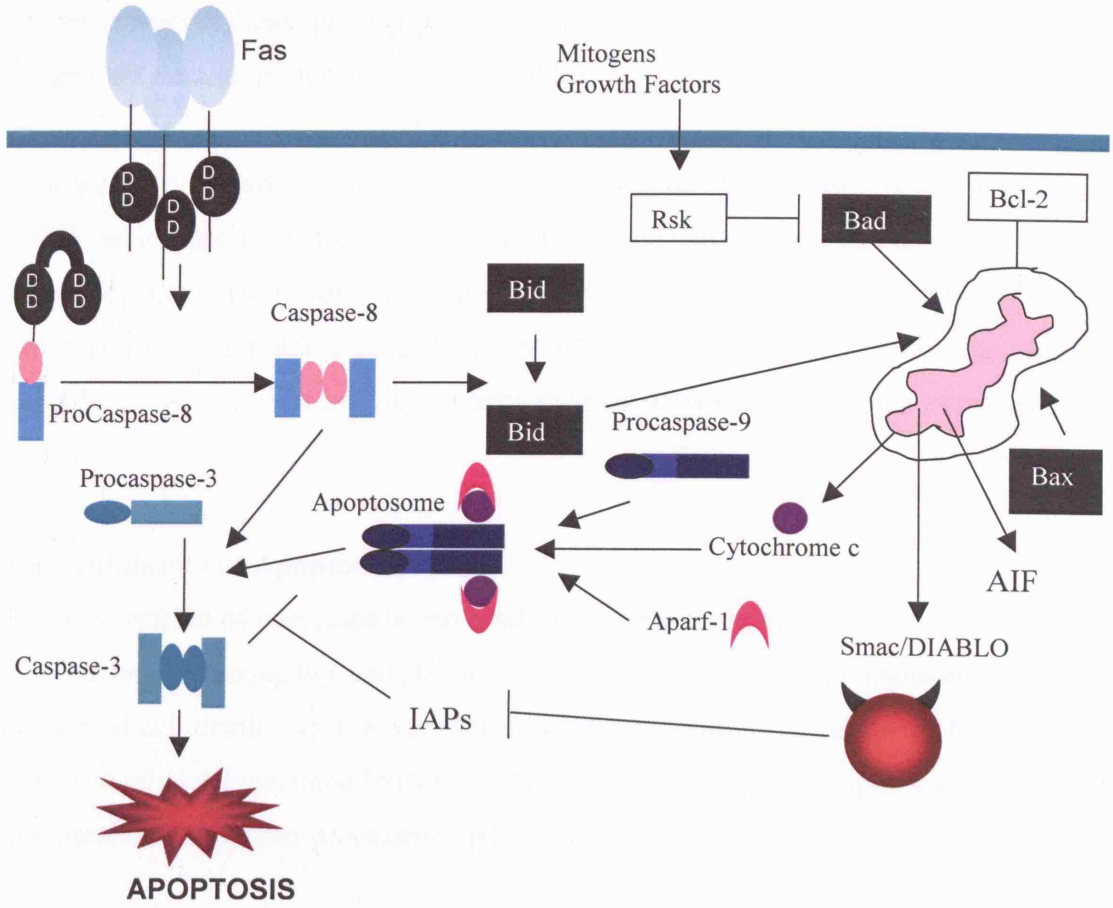

Figure 1.17 Activation of apoptosis through the mitochondrial pathway. Extracellular signals can have an effect on the relationship of Bcl-2 family members at the surface of mitochondria. Pro-apoptotic Bcl-2 proteins can release a variety of molecules from the mitochondrial compartment. Cytochrome c is considered to be the primary mitochondrial factor in caspase-mediated apoptosis. Together with Apaf-1 and procaspase-9, cytochrome c forms the apoptosome, which is a potent activator of caspase-3. Second mitochondria activator of caspase (Smac/Diablo) is a mitochondrial factor that can inhibit the action of inhibitor of apoptosis proteins (IAP), which themselves can prevent caspase-3 activation and action. AIF is also released from mitochondria and it can activate apoptosis via unknown, caspase-independent pathway. (Adapted from Wyllie *et al.*, 1980; Morita and Tilly, 1999).

1.6.4 The Caspase Family

Caspases are a family of cysteine proteases; they are the main death proteases of the cell. Morphological changes seen in apoptotic cells are the result of cleavage of cytoskeletal proteins and nuclear lamins by caspases, (Kerr *et* al., 1972; Rao *et al.,* 1996; Kothakota *et al.,* 1997; Buendia *et al.,* 1999). These proteases form a large family that consists of at least fourteen members (Budihardjo *et al.* 1999). They all show a high degree of specificity, which is important in apoptotic cell death as the

process involves cleavage of a particular group of proteins in a coordinated manner, rather than random proteolysis (Grutter 2000).

Caspases are initially present as inactive zymogens, procaspases (Earnshaw *et al.* 1999), which are activated by proteolysis. Consequently, once caspase activation is triggered, the effect can be exponentially multiplied by processing of other procaspases to their active state. This type of activation has been termed 'the caspase cascade', it is used extensively for activation of downstream effector caspases-3, -6 and -7.

1.6.5 Inhibitors of Apoptosis Proteins

When activation of one caspase can easily escalate into the recruitment of the caspase cascade and subsequently cell death, there must be preventative measures to avoid accidental cell death due to adventitious caspase activation (Deveraux & Reed 1999). A family of proteins named IAPs have the ability to bind to procaspases and activated caspases, blocking their processing and activity (Figure 1.17).

IAPs also have their own controlling factor termed second mitochondria-derived activator of caspases (Smac) or Diablo that can prevent IAPs from binding caspases, allowing caspases to be activated and perform their part in the apoptotic program (Verhagen *et al.,* 2000). Smac/Diablo is normally confined to the mitochondria, but once released, it binds to IAPs and removes this block in the cell death pathway. Furthermore, Smac/Diablo interestingly possesses an amino-terminal sequence that is capable of procaspase-3 activation (Chai *et al.,* 2000).

One of the end points in apoptosis is DNA fragmentation into multiples of approximately 180 bp (Wyllie *et al.,* 1984). Recently, the enzyme responsible for this action has been found, and it is now termed caspase-activated DNase (CAD) (Enari *et al.* 1998). CAD is found as an inactive complex, which is bound to an inhibitory subunit, inhibitor of CAD (ICAD) (Sakahira *et al.,* 1998). The finding that activation of CAD is dependent on caspase-3-mediated cleavage of ICAD provides the final link between the programmed cell death pathway and internucleosomal DNA cleavage. The active caspase-3 molecule cleaves the inhibitory subunit, which then results in the

release of the catalytic enzyme. Subsequently, the activated CAD proceeds with its intended mission to cleave the genomic DNA.

1.6.6 Apoptosis and Tissue Homeostasis

The maintenance of tissue homeostasis is finely balanced between cell proliferation and apoptosis. The maintenance of this balance is crucial, too much proliferation and too little apoptosis leads to fibrosis and to the anatomical and physiological problems that are associated with it. If apoptosis supersedes proliferation, the result is a reduction of the tissue mass. If the process runs out of control, it eventually reaches a point where physiological function is no longer possible (Thompson, 1995).

1.6.7 Potential Defects with the Machinery of Apoptosis in Keloid Scars

Keloid scars have previously been proposed to have faults in their apoptotic machinery. A number of studies examining specific components of the apoptotic machinery have examined whether keloid scar tissue and cells possess defects which may affect their ability to undergo apoptosis. Keloid scar-derived fibroblasts and tissue show higher basal levels of TNF-receptor-associated factors: TRAF1, TRAF2, TNFα, IAP-1, and NFκB, which are all anti-apoptotic genes, compared with normal dermal fibroblasts (Messadi *et al.*, 2004). Keratinocytes derived from keloid tissue have also been found to signal normal skin fibroblasts to up-regulate their ERK and JNK phosphorylation along with their expression of Bcl-2 and TGF-β1 (Funayama *et al.*, 2003) all of which are linked with cell survival and growth. Further, keloid scar-fibroblasts over express IGF-1 receptor, which could also protect keloid scar-fibroblasts from undergoing apoptosis (Ishihara *et al.*, 2000).

There have been controversial and unconvincing results with regards to the expression of the caspase and the bcl-2 family of genes in keloid scars. Inactivity of caspases-3, -8 and -9 in keloid scar-derived fibroblasts after Fas-mediated and staurosporine-induced apoptosis has been reported (Chodon *et al.*, 2000). In contrast, Akasaka *et al* (2000) report increased expression levels of caspase-3 and -2 by immunohistochemistry of keloid scar tissue compared to that in normal dermal tissue, suggesting increased apoptosis. In addition, deregulated Bcl-2 and p53 activity, as well as p53 gene mutations have been reported in keloid scar cells and tissue (Ladin *et al.*, 1998; Saed *et al.*, 1998). With respect to the expression level of Fas, Bcl-2 and

Bax, again conflicting evidence exists, where either keloid scar and normal dermal fibroblasts display no differences (Chodon *et al.*, 2000), or intense staining of Bcl-2 is found in keloid scar tissue compared to that of normal dermal tissue (Toefoli *et al.*, 1999). Nevertheless, the apoptosis induction mechanisms used by previous research groups are not ideal, having no specific relevance to the apoptosis that takes place during wound healing. Furthermore, research on keloid scar tissue and keloid scar fibroblasts themselves should not be confused, as fibroblasts in culture could behave differently from those in the *in vivo* situation, due to paracrine signalling and interaction with the extracellular matrix. In respect of this, no clear consensus exists on whether keloids display a fault in their apoptosis machinery. It is acknowledged that the work in this thesis is carried out on fibroblasts alone *in vitro* rather than *in vivo* with the added effects of many other cell types and paracrine signalling. The purpose of this was to determine the response of specifically fibroblasts to wound healing apoptosis cues, then later, to progress investigations to an *in vivo* animal wound model.

1.7 Evidence of Actual Defects in the Apoptosis of Keloid Scar Fibroblasts

"Pathological scars occur when the normal tissue repair process becomes deregulated and the forming scar tissue is seemingly trapped in the proliferative phase of wound healing".

Desmouliere et al., 1995

Under normal circumstances, the fibroblasts that have migrated into the wound area, to proliferate and differentiate into their 'active' phenotype (particularly myofibroblasts) begin to undergo apoptosis on day 12, peaking at day 20 and resolving by day 60 (Greenhalgh, 1998). This mechanism ensures that the cells brought in to repair dermal damage and thus produce a scar are triggered, after becoming redundant, to undergo cellular suicide by as yet uncertain mechanisms. Desmouliere *et al* (1995) proposed therefore that a finite balance of cell proliferation and cell clearance by apoptosis must exist to result in a pale, flat relatively normal quiescent scar.

"The prolonged presence of excessive numbers of myofibroblasts, which is characteristic and potentially the cause of keloid scars, could theoretically be caused by either unremitting induction of these cells or their failure to undergo apoptosis".

Appleton et al. 1996

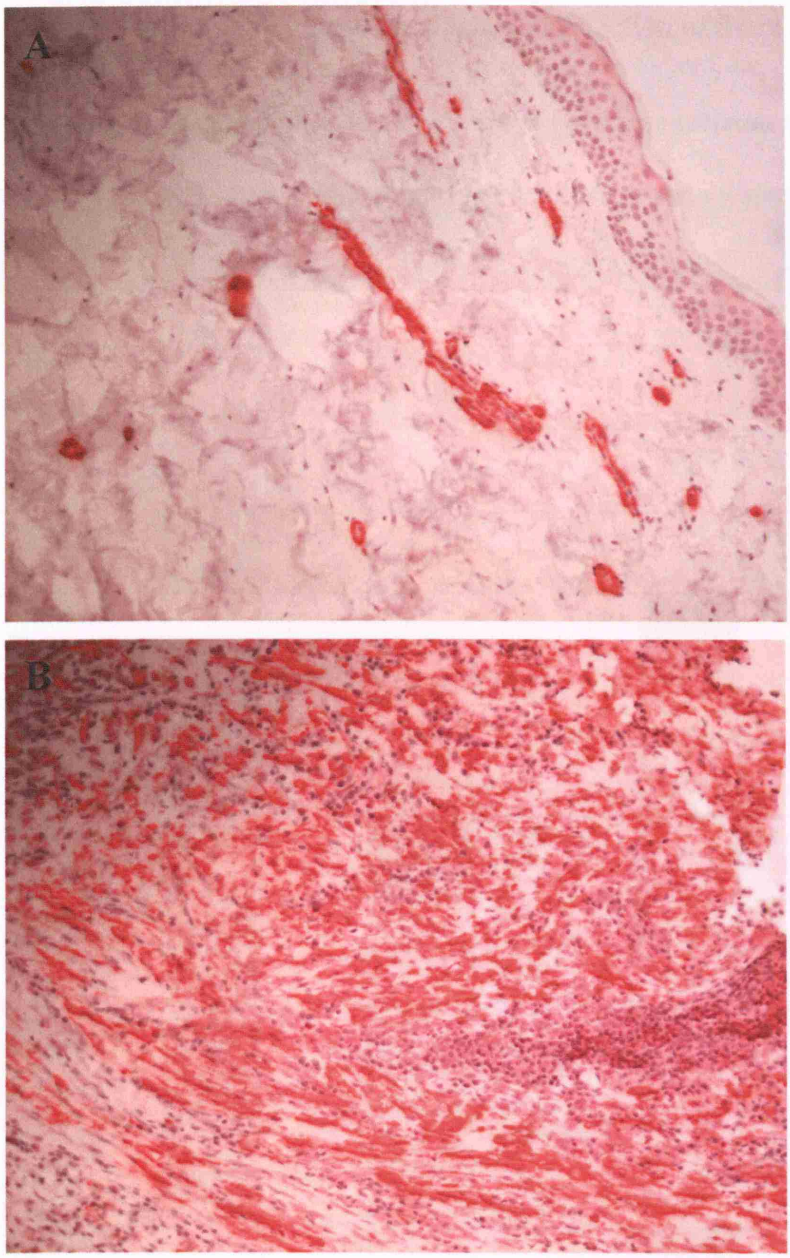

Figure 1.18 α-smooth muscle actin staining of normal scar (A) and keloid scar (B) tissue sections. The normal scar tissue only expresses red (α-SMA) staining around blood capillaries, presumably of pericytes, whereas keloid scar tissue expresses α-SMA staining of myofibroblasts throughout large areas of the tissue (x100 Mag). (Vigor C, unpublished data).

Figure 1.18 clearly illustrates the relatively quiescent nature of normal scar tissue, which contains very few cells in the scar area in comparison to the very active nature of keloid scar tissue, which expresses vast amounts of myofibroblasts and inflammatory infiltrate. A number of research groups have attempted to determine if keloid scars exhibit a fault in apoptosis induction. So far there has only been weak and/or contradictory evidence published (a selection of these is given in Table 1.4).

Table 1.4 Previously published data on attempts to investigate apoptosis in keloid scars

Publication	Apoptosis in tissue data			Methods	Apoptosis in cells data			Methods
	Keloid scar	Normal Scar	Dermis		Keloid scar	Normal scar	Dermis	
Akasaka *et al.*, 2000				Caspase 2 and 3				Serum-starvation
Akasaka *et al.*, 2001				Tunel				
Sayah *et al.*, 1999				Tunel and apoptosis gene array				
Messadi *et al.*, 1999								Anti-Fas antibody, TNFα
Ladin *et al.*, 1998				Tunel				Serum starvation, hydrocortisone, γ-IFN and hypoxia
Appleton *et al.*, 1996				Apoptotic bodies				
Luo *et al.*, 2001				Tunel, radiation, serum starvation				
Chodon *et al.*, 2000				Fas and staurosporine-induced apoptosis. Caspase 3, 8, 9 TGF-β1 inhibition				
Ishihara *et al.*, 2000								Ceramide-induced apoptosis, Tunel, flow cytometry
Chipev *et al.*, 2000								Serum starvation, Annexin V and Tunel
Key	= Increased Apoptosis = Less Apoptosis			= Both increased and decreased apoptosis depending on stimulus				

Research has been carried out on both tissue and fibroblasts derived from keloid scar, normal scar and normal dermis. Some groups found the level of apoptosis occurring in keloid tissue reduced compared to that observed in normal dermis or normal scar tissue (Ladin *et al.* 1998; Messadi *et al.* 1999; Chipev *et al.* 2000; Sayah *et al.* 1999; Luo *et al.* 2001; Chodon *et al.* 2000), whilst other groups found that it was actually increased (Appleton *et al.* 1996, Akasaka *et al.* 2000, 2001).

Comparisons of apoptosis levels in keloid scar-derived tissue and cells in comparison to that of normal scar or dermis still remains inconclusive. These inconsistent results are undoubtedly due to differences in experimental design, age of scar tissue and methods used to induce apoptosis. It may be misleading to consider the level of apoptosis occurring in the tissue of mature scars since it is likely to be the early events of scar formation that are of most importance. The maturity of scars used in the published studies varies dramatically. For instance, a study by Sayah *et al* (1999) investigated keloid scars between 3-10 years old, in contrast to a study by Chen *et al* (2003b) assessing gene expression levels of keloid scars, which were 7-16 months old; many studies however do not state the age of the scars used. Methods employed by research groups to induce apoptosis of cultured cells were varied including: Fas antibody, TNF-α or serum withdrawal, most of which have no known relevance to the apoptotic induction events that occur during wound healing. In addition, it has been reported that normal cultured primary fibroblasts are actually resistant to apoptosis induction following serum withdrawal, irradiation or loss of adhesion to the ECM (Frisch and Francis, 1994; McKenna *et al.* 1996; Mcgill *et al.* 1997), methods which were used in a number of these studies comparing the level of apoptosis induction in keloid scar versus normal tissues.

In respect of this therefore, these experiments would only detect whether cells exhibit a defect in the process of apoptosis as a whole rather than the specific events regulating the apoptosis induction which heralds the transition between the proliferative and the remodelling phases of wound healing.

Furthermore, most of the previously published studies that attempt to investigate apoptosis of keloid scarring have compared keloid scar-derived fibroblasts with those of normal dermis. This so-called 'normal control' is not ideal, since these cells are not

from a previously wounded site and as such their predisposition to either a keloid-like phenotype or that of a normal scar is unknown. Furthermore, the very process of undergoing wound healing may irretrievably change the phenotype of the cells. In addition, the origin of the wound cells is yet to be confirmed, cells may not necessarily be of the same origin, it is possible that they have migrated in from near-by tissue or are circulating progenitor stem cells, if this is the case then the inherent response of wound cells would clearly be different from that of cells derived from normal dermis. Taking these possibilities into consideration the research presented in this thesis involves using fibroblasts derived from normal scar as a more appropriate control.

1.8 Research Rationale

Research presented in this thesis therefore examines the induction of apoptosis using *in vitro* models that mimic certain aspects of wound healing, potentially being more relevant to the events that take place during the final stages of wound healing, ending the fibroproliferative phase. Keloid scar-fibroblasts were compared to fibroblasts derived from normal scars as a more reliable control to dermal fibroblasts. Attempts were made to determine the crucial events involved in wound-healing related apoptosis, working out their interplay by dissecting specific mechanisms and potentially determining their fundamental importance (if there is one) to keloid scarring. Critical observations have recently given clues as to the mechanisms involved in the specific form of apoptosis induced during wound healing. Fluck *et al* (1998) and Buckley *et al* (1999) have provided evidence for the role of fibrillar collagen and extracellular matrix fragments in the induction of fibroblast apoptosis, which may relate to that which takes place during wound healing. These findings have thus provided the framework for the research presented in this thesis.

1.9 Research Aims

Section A

1) To determine whether keloids exhibit an inability to undergo apoptosis in general (chemical induction) or specifically to wound healing apoptosis induction mechanisms (matrix contraction and remodelling).
2) To investigate the mechanisms involved in wound healing-induced apoptosis (that of collagen matrix contraction) and where faults may lie with keloid scars.

Section B

3) To investigate whether the myofibroblast phenotype is exclusively involved in the specific mechanisms of wound healing-induced apoptosis.
4) To determine what role TGF-β1 plays in the apoptosis that takes place during wound healing.

Chapter 2

Methods and Materials

2.1 Methods and Materials

Unless otherwise specified all tissue culture materials were supplied by Gibco and Sigma, tissue culture plastics by Greiner and Fisher and all chemicals used in this study were the best grade available supplied by Sigma, BDH and Fisher. All solvents were analytical (AnalaR) grade supplied by BDH. Details of other suppliers of chemicals or compounds used are noted as and when appropriate within the methods text.

Primary fibroblast cell-strains were obtained from patients undergoing surgical excision, local ethical approval was obtained (ethical approval number EC2002-20) along with patient consent. Patient details including: age, sex and body site are given in Table 2.1. All cell culture work was carried out in sterile class II laminar airflow hoods, (Laminar HB2448, Heraeus Instruments) and cells maintained in a Galaxy S incubator (Wolf Laboratories) at 37°C, humidified with a CO_2 concentration of 5%.

Table 2.1 Cell strains used in this study

Scar Type	Age	Male:Female Ratio	Origin
Keloid Scar	29.6 ± 11.	3/6	6 from the ear, 1 from the neck and 2 from the chest
Normal Scar	23.3 ± 9.3	3/5	1 from the buttock, 3 from the chest, 3 from the abdomen and 1 from the face
P-value of Keloid vs Normal scar	P=0.232		

It was noted that although all keloid scar-derived fibroblasts behaved in a distinct fashion from the normal scar-derived fibroblasts irrespective of the body site source, ideally it would have been better to try to more closely site match the keloid scars with the normal scars. However, the preponderance of keloid scars removed at Mount Vernon Hospital over this time-period was from ears and few normal scars need revision when on ears.

2.2 Primary Human Tissue Culture

Routine tissue culture was performed in normal growth media (referred to as 10% NGM in the text) and was made up as follows: 90% Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Calf Serum (FCS), 7mM 1M HEPES (pH 8), 1000 unit/ml Penicillin/Streptomycin and 2mM L-Glutamine.

The volume of FCS was altered accordingly, depending on the growth requirements of individual experiments, for instance; 1% FCS (referred to as 1% NGM in the text). Culture media was stored at 4°C, used within 4 weeks and warmed in a 37°C water bath prior to use.

2.2.1 Isolation and Propagation of Cells

Cells were isolated from human skin samples, which would otherwise have been discarded, from elective surgical procedures at Mount Vernon Hospital after informed consent had been obtained.

The epidermis and fatty tissue were removed using sterile forceps and a scalpel. The dermis was then cut into approximately $1mm^2$ pieces and then placed into $25cm^2$ adherent tissue culture flasks with 5mls of 10% NGM. The flasks were incubated at 37°C for approximately 3 weeks; refreshing the media twice a week until approximately 80% of the surface area was covered with fibroblasts (80% confluent).

The tissue pieces were then removed, and the dishes washed twice briefly with warmed versene. 1ml of 1:10 solution of trypsin:versene (final enzyme concentration of 0.25%) was added to the flasks, which were incubated at 37°C, until the cells had detached. The trypsin solution was neutralised with the addition of 4mls 10% NGM. The cell suspensions were pelleted by centrifugation for 5mins at 1000xg and re-suspended in fresh 10% NGM, and sub-cultured on a split ratio of 1:3.

Media was refreshed twice weekly and cells were further sub-cultured routinely in a split-ratio of 1:3, by trypsinisation (as described above) on confluency. Only cells of passages 1-8 were used for experimentation.

2.2.2 Cryogenic Storage.

Exponentially growing cells were trypsinised, pelleted by centrifugation (1000xg, 5mins) and re-suspended in 'freezing medium' (9ml FCS and 1ml Dimethyl Sulphoxide (DMSO)). Typically a confluent 75cm^2 adherent tissue culture flask was re-suspended in 3mls of freezing medium, which was then aliquoted into cryovials (1ml per cryovial). Cryovials were slowly frozen by placing insulated into a -80°C freezer for 24hrs before being transferred to liquid nitrogen for long-term storage.

Cells were retrieved from cryogenic storage by rapidly thawing the cryovials in a 37°C water bath. The cell suspension was immediately transferred to a 15ml labeled centrifuge tube. To this 9mls of 10% NGM was added slowly, gently agitating the solution. The cells were then centrifuged (5mins at 1000xg), re-suspended in 10mls of fresh 10% NGM, transferred to 75cm^2 adherent tissue culture flasks and cultured as described in section 2.2.1

2.2.3 Determination of Cell Number and Viability

Cells were stained with Trypan Blue to determine viability (an aliquot of a well-mixed cell suspension was diluted 1 in 1 with Trypan Blue (0.4%, Sigma.) The cells were counted using a haemocytometer, which was examined under an inverted phase contrast microscope (Olympus CK2, Olympus Optical Co), where dead cells were seen to stain darkly with the Trypan Blue.

2.3 Three-Dimensional Cell Culture

2.3.1 Three-Dimensional Collagen Matrix Culture of Fibroblasts

2.3.1.1 Rat Tail Collagen Preparation

Sprague Dawley Rat-tails (kind present from the Gray Laboratories, Cancer Research UK) were soaked in ethanol prior to dissection. Tendons were dissected from rat-tails under sterile conditions. The tendons were chopped finely and suspended in 0.1% (v/v) acetic acid (300ml/g of tendon) for 3-days at 4°C with stirring. The solution was then centrifuged for 60mins at 10,000xg. The supernatant was kept and pellet discarded. Gamma ray irradiation for 1hr (^{60}Cobalt Gamma-sterilisation for 1hr, 170Gy) was used to sterilise the final collagen solution. This solution was aliquoted out into 50ml sterile tubes and stored at 4°C until use.

2.3.1.2 Examination of Collagen Concentration for Each Batch

In order to assess how standardised the rat-tail collagen preparations were, the total protein content of each batch was assessed. In this manner, collagen solutions of approximately the same concentration were used throughout.

160µl of each batch was aliquoted into a well of a 96-well tissue culture plate, to which 40µl of a total protein dye (BioRad protein assay, 500-0006) was added. The solutions were mixed by pipette action, making sure not to introduce any air-bubbles. Once thoroughly mixed, the lid was placed on the 96-well tissue culture plate (Falcon 35-3072) and the absorbance read at 595nm, using a microplate reader (BioRad Model 550). The optical density (OD) of each batch was then plotted, see Figure 2.1.

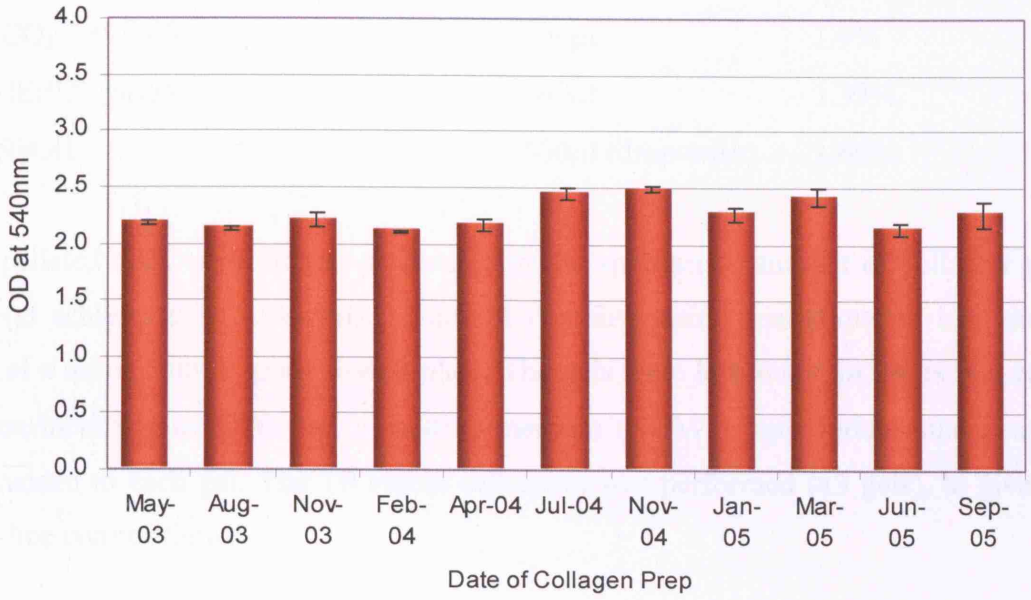

Figure 2.1 Total optical density (OD) representing protein concentration, of each rat tail collagen preparation used throughout the time period of experimentation. Measurements were made prior to using the collagen prep with BioRad Total Protein Dye (methods 2.3.1.2). All preparations were relatively constant, only varying between 2 and 2.5 OD (arbitrary units), with no statistical difference between preps.

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2.3.1.3 Preparation of Collagen Gel Matrices

The volumes used in the following method were altered as appropriate to the number of collagen gels required for a specific assay ($3x10^5$ cells were required per 1.5ml gel).

Cell cultures were trypsinised and the cell number determined using a haemocytometer. An appropriate aliquot of the cell suspension was taken and pelleted at 1000xg for 5mins. Triplicate gels were prepared for each different experimental variable (treatments and time-points). Triplicate gels were also made for a time zero (T0) cell count as a baseline control. During cell pelleting, the appropriate volume of collagen gel mix was made up on ice as follows (adapted from Bell *et al.*, 1979):

Example for 40mls of collagen solution:

Ingredient	*Volume*	*Final Concentration*
Rat Tail Collagen	36mls	85.5%
Minimum Essential Medium Eagles (MEM x 10)	4mls	9.51%
$NaHCO_3$ (11% w/v)	800µl	1.9%
1M HEPES (pH8)	560µl	1.33%,
1M NaOH	500µl (drop-wise)	1.66%

The pelleted cells were then re-suspended in the appropriate amount of collagen gel mix (to achieve $2x10^5$ cells/ml). 1.5mls of this suspension was aliquoted into each well of a tissue culture grade 6-well plate. The gels were left to set for 24hrs in a cell culture incubator at 37°C. lml of culture medium (NGM or appropriate other), was then added to each gel. The T0 viable cell count was performed (x3 gels), to give a base-line control figure.

At day-4, a second viable cell count was performed from an appropriate number of anchored gels (x3 per treatment group). The culture medium was refreshed in the remaining gels. Gels were either retained anchored (adhered to the plastic) or released by running a scalpel blade around the edge of the gel ensuring that it floats freely. At day-7 (Figure 2.2) viable cell counts were performed on cells harvested from the remaining collagen gels.

Viable cell counts were performed by harvesting cells from the gels using a collagenase-D solution (0.05% (w/v) collagenase D (Roche), 0.5% (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS with Ca and Mg). The gels

were placed into fresh 15ml centrifuge tubes containing 1ml of the collagenase-D solution per gel. These were placed in a 37°C shaker for approximately 30mins (until the gel completely dissolved). Once dissolved, 5mls of PBS (with Ca and Mg) was added per tube. The cells were collected by centrifugation at 1000xg for 5mins, and then re-suspended in Trypan Blue solution (as in section 2.2.3) and viable cell counts performed.

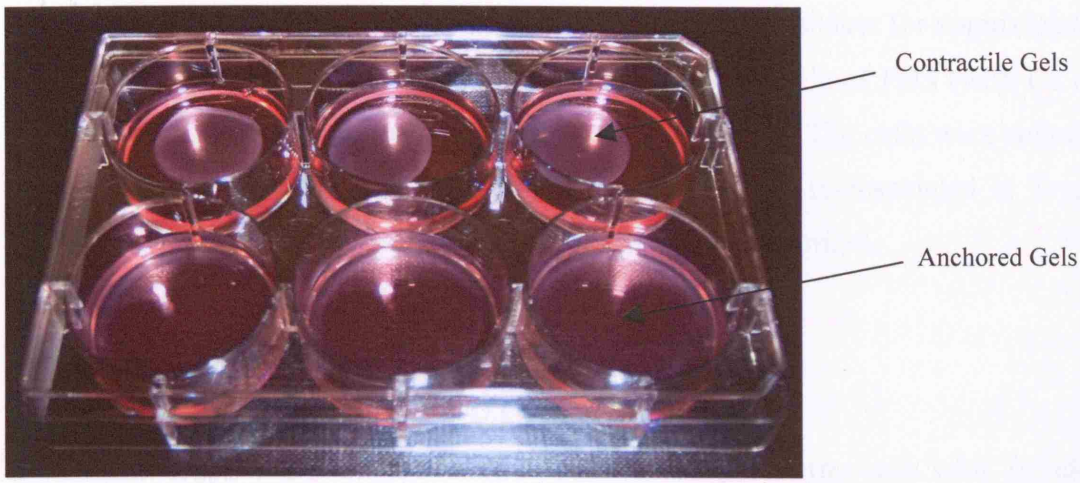


Figure 2.2 Photograph of day-7 anchored and contractile collagen gels. Triplicate gels for each treatment group were set up in 6-well tissue culture plates.

2.3.2 Three-Dimensional Fibrin Matrix Culture of Fibroblasts

The volumes stated in the following method, were altered according to the number of fibrin gels required for a specific assay. A cell suspension was obtained as in 2.3.1.3, but instead of re-suspending them in a collagen gel solution they were re-suspended in a fibrin gel solution.

To make the fibrin gel solution 2mg/ml of fibrinogen (Sigma Type IV, F-4753) was weighed out and dissolved in SFM (serum free medium, DMEM) then filter sterilized with a 0.2μm filter (Nalgene). The solution was stored on ice until use. Pelleted cells were re-suspended in the fibrin gel solution to give $2x10^5$cells/ml. This solution was then aliquoted into a 6-well plate, 1.5ml/well. 1.25IU of freshly thawed thrombin (Sigma T-1063, at 0.83IU/ml) was added to each fibrin gel aliquot. The fibrin aliquots were gently agitated in their wells to allow mixing of the thrombin. The gels were left to polymerise for 24hrs in a 37°C culture incubator. As with the collagen gels, culture media was then added and a T0 cell count was performed. At day-4 of the culture

period cell counts were performed from the appropriate anchored gels, the media was refreshed on the remaining gels and appropriate gels were loosened to allow contraction. At day-7, cell counts were performed on the remaining gels.

In order to perform cell counts, cells were harvested from fibrin gels using a trypsin solution (0.25% (w/v) trypsin, 0.1% (w/v) ethylenediaminetetacetic acid (EDTA) in PBS without Ca and Mg). Gels were placed in separate 15ml centrifuge tubes with 1ml of trypsin solution per gel. These were placed in a 37°C shaker for approximately 20mins (until gel completely dissolved). Once dissolved, 5mls of PBS (with Ca and Mg) was added per tube to neutralise the enzymatic activity. The cells were collected by centrifugation at 1000xg for 5mins; the pellet was then re-suspended in Trypan Blue solution (as in section 2.2.3) and viable cell counts performed.

2.4 In Situ 3-Dimensional Cell Staining

2.4.1 Toludene Blue Staining of 3-D Gels.

Cell morphology within 3-D matrices was assessed by staining cells with Toludene blue (No. G298, Gurr's Ltd) in the following manner.

Rather than harvesting cells from 3-D matrices at day-7 (at the end of the gel culture period), intact gels were washed once with PBS (without Ca and Mg) and fixed in 5mls of 10% formal saline overnight at room temperature. The following day the gels were washed 3 x with PBS then stored at 4°C in PBS, until needed.

The gels were placed into 15ml small centrifuge tubes in a 1% solution of Toludene Blue and incubated at room temperature for approximately 15mins on a rotary shaker. The gels were washed thoroughly 3 x with distilled water (dH_20), then placed on a microscope slide with a cover-slip and examined by light microscopy (Nikon eclipse T5100).

2.4.2 Haematoxylin Staining of 3-D Gels

Staining was carried out as above (2.4.1), but using a 1% solution of Harris Haematoxylin (BDH, 351945S) and Eosin (BDH, 341973R) instead of a 1% solution of Toludene Blue.

2.4.3 Staining Fibroblast Populated Collagen/Fibrin Matrices for α-SMA

To assess the myofibroblast content in 3-D matrices, gels were harvested at day-7 of matrix culture and stained as follows:

All gels were loosened in their 6-well plates so that they were free floating. The medium was aspirated and replaced with 5 ml of 10% formal saline per gel. The gels were left overnight to fix at room temperature. The following day the gels were washed 3 x with PBS then stored at 4°C in PBS, until use.

Just prior to staining, the PBS was replaced with ice-cold methanol (to permeabilise the cell membranes) and incubated for 1hr at -20°C. The matrices were then washed 3 x with PBS for a further 1hr on an orbital shaker (Luckham R100, Rotatest Shaker).

The gels were then incubated in 1ml of dilute primary antibody, mouse monoclonal anti-α-SMA antibody (Sigma, clone 1A4) diluted 1:1000 with tTBS (Tween + Tris buffered saline) [1 x TBS (10mM Tris-base, 13.8mM NaCl, 2.7mM KCL) pH7.4 + 0.05% Tween-20]. Separate gels used as negative controls were incubated in the same way but with 1ml of tTBS alone. The gels were left to incubate overnight at room temperature. The gels were then washed in their wells 3 x with PBS for a total of 1hr before adding the secondary antibody, rabbit anti-mouse FITC-conjugated (Dako, diluted 1:400) with Propidium Iodide as a nuclear counter-stain (25μg/ml). The gels were left to incubate for 1hr at room temperature in the dark to prevent photo-bleaching of the fluorescence.

The gels were finally washed 6 x with PBS for 2hrs in total, in the dark at room temperature. The gels were then carefully placed on a slide with 2 drops of Vectashield (anti-fade reagent, Vector H-1000) and a cover-slip placed on top, then immediately viewed with fluorescence microscopy (Zeiss Fluorescence Microscope, Germany). Myofibroblasts, which were positive for α-SMA demonstrated intracellular stress fibers that fluoresced bright green. Negative cells showed red nuclear staining only.

2.5 Measurement of Matrix Contraction

In order to measure the degree of contraction induced by fibroblasts, gels *in situ* within the 6-well plates were photographed using a camera system (UVP Bioimaging System, Epi Chemi II Darkroom), which was linked up to a computer. The degree of matrix contraction was measured using Lab Works 4.0, (UVP, Bioimaging Systems). By measuring the gel circumference and the circumference of the culture well (the original circumference of the gel), the degree of contraction could be worked out as the percentage change in matrix circumference from that at day-0 (where matrices were attached to the edges of the culture well).

2.6 Assay of Cell Proliferation on Matrix Protein-Coated Plates

2.6.1 Preparation of 96-Well Plates with Fibronectin and Collagen Coatings

The required amount of fibronectin (Sigma, stock at 1mg/ml) and collagen type I (First Link, stock at 2mg/ml) was diluted in PBS (with Ca and Mg) from their stock solutions to give a concentration of 10μg/ml. The solutions were then stored on ice, before adding 50μl/well of either fibronectin or collagen type I solution to appropriate wells of 96-well tissue culture grade plates. Tissue-culture plates were then incubated in a 37°C tissue culture incubator for 4hrs.

The appropriate number of cells for the experiment were collected by routine trypsinisation of cultures, counted and centrifuged at 1000xg for 5mins. The supernatant was then removed and the cell pellet re-suspended in the appropriate volume of culture media, to give $2.5x10^5$cells/ml. Once the extracellular matrix protein solutions had sufficiently coated the wells of the 96-well plates, the wells (including the non-coated control wells) were washed 2 x with 100μl/well PBS (with Ca and Mg) and 200μl/well ($5x10^4$cells/well) of the cell suspension was added. Triplicate wells were seeded for each treatment group, and separate 96-well plates were set up for each time-point of: 24hrs, day-3 and day-6. Cell number was measured in harvested plates by Crystal Violet Staining.

2.6.2 Crystal Violet Staining

At the specified time points media was gently removed from each well using a multi-channel pipette and replaced with 100μl/well of crystal violet solution, (0.5% crystal

violet, 0.85% NaCl, 5% formal saline, 50% ethanol in dH_20) then incubated for 10mins at room temperature. This solution was then removed and the plate washed 3 x with PBS and thoroughly drained. 100µl of 33% acetic acid was then added per well to elute the dye from the cells. The optical density was then read using a microplate reader at 595nm. Cell-free wells were used as intra-plate background control measurements, which were subtracted from all well measurements for that plate.

2.7 Protein Detection Methods

The protein detection methods used included: detection of specific proteins using immunostaining of western blots, detection of total protein banding patterns using Silver Staining and detection of MMP activity using Zymography.

2.7.1 SDS Polyacrylamide Gel Electrophoresis

2.7.1.1 Protein Sample Preparation from Monolayer Cultured Cells

Cells cultured in $75cm^2$ adherent tissue culture flasks were washed 2 x with PBS (without Ca and Mg), and drained. 500µl of sodium dodecyl sulphate (SDS) lysis buffer (0.1% SDS, 125mM Tris pH6.8, 1mM Phenylmethylsulphonylfluoride (PMSF) was added to each flask, making sure all cells were coated in the lysis buffer. The flasks were left on ice for 15mins, gently agitating every 5mins. Cells were then harvested with a cell scraper and pipetted into labeled microfuge tubes. Following this, 500µl of 2 x Laemmli reducing sample buffer (4% SDS, 120mM Tris pH6.8, 0.01% Bromophenol blue, 10% Glycerol, 2% 2-mercaptoethanol) was added to each protein lysate. The cell suspensions were then heated for 3mins at 97°C and centrifuged at 1300xg for 3mins before storing at -20°C. Just before use the samples were thawed at room temperature.

2.7.1.2 Protein Sample Preparation from 3-D Matrix Cultured Cells

At day-7 of the gel culture period triplicate gels of the same treatment were pooled and homogenised in 300µl of SDS lysis buffer (see 2.7.1.1 above) using a mortar and pestle. The homogenised suspensions were then further broken up by passing through a 27G hypodermic needle (Terumo, Neolus 27G). 500µl of the homogenised suspensions were aliquoted into microfuge tubes, to which 500µl of 2 x Laemmli reducing sample buffer (see 2.7.1.1 above) was added. The cell suspensions were then

heated for 3mins at 97°C and centrifuged at 1300xg for 3mins before storing at -20°C. Just before use the samples were thawed at room temperature. There was no need to assess equal cell number in each protein sample preparation as each preparation was derived from equally seeded collagen gels. In addition, the experiment was carried out to assess the different levels of protein being produced by cells in response to different growth environments.

2.7.1.3 SDS Gel Formulation

Depending on the size of the protein of interest either a 10% or 15% separating gel was cast.

	10% Separating gel	**15% Separating gel**
30% Acrylamide (Sigma)	5mls	7.5mls
1M Tris pH8.8	5.6mls	5.6mls
dH_2O	4.35mls	1.85mls
10% SDS	0.150mls	0.150mls
10% APS (Ammonium persulfate)	50µl	50µl
TEMED (Invitrogen)	10µl	10µl

5% Stacking gel

30% Acrylamide (Sigma)	0.835mls
1M Tris pH6.8	0.625mls
dH_2O	3.515mls
10% SDS	0.050mls
10% APS	25µl
TEMED (Invitrogen)	5µl

The Mini protean III kit (BioRad) was used for SDS-PAGE and set-up according to supplier's instructions. The SDS gels were cast with a 5% stacking gel. Once polymerised, the comb was gently removed, and the glass plates assembled within the gel running tank with 1 x running buffer [10% x10 stock, (0.025M Tris base, 0.192M Glycine, 0.1% SDS in dH_2O) in 90% dH_2O]. 10µl of precision plus pre-stained blue protein marker (BioRad, 310000326) was loaded first as a molecular weight marker.

Then 25µl per lane of the protein samples were loaded. The gels were run at 200 volts for 60mins or until the dye front had run to the end of the gel, depending on the migration pattern of the protein of interest.

2.7.2 Western Blotting

Once the proteins had been successfully electrophoresed, the gels were briefly pre-soaked in fresh transfer buffer, [10% x10 stock, (25mM Tris base, 192mM Glycine in dH_2O) 20% methanol, 70% dH_2O] as were six pieces of pre-cut filter paper (Whatman 3MM) and one pre-cut nitrocellulose membrane (Amersham Biosciences, Hybond-ECL) per gel. Three filter pads were placed onto the base (anode) of the semi-dry western blotter (BioRad), followed by the nitrocellulose membrane, the gel and then three final filter pads; taking care to remove any air-bubbles.

The lid (cathode) was attached and protein transferred for 45mins at 25V. The nitrocellulose membrane was then placed in blocking buffer prior to antibody detection (section 2.7.2.1) and the gel was stained with Coomasie blue (0.25% Brilliant Blue R-250 (Sigma), 40% methanol, 10% acetic acid in dH_2O) on a rotary shaker for 40mins. Excess dye was removed by placing the gel in destain solution (40% Methanol, 10% Glacial Acetic Acid in dH_2O) and incubating overnight at room temperature on the rotary shaker. The stained gel was then viewed over a light box to confirm that samples had transferred successfully.

2.7.2.1 Antibody Detection of Proteins

The nitrocellulose membrane was blocked in blocking buffer [5% BSA, 0.1% Tween in 1xTBS (10mM Tris-base, 13.8mM NaCl, 2.7mM KCL)] for 2hrs on a rotary shaker at room temperature. BSA was used in the blocking buffer instead of dried milk powder as results were consistently cleaner.

Following blocking of non-specific binding, the membrane was washed 3 x for 15mins total in tTBS wash buffer with shaking. The membrane was then incubated in the relevant primary antibody (diluted in blocking buffer as detailed in Appendix II) overnight at room temperature on the rotary shaker.

The membrane was washed 3 x for 15mins total in fresh tTBS wash buffer. The secondary antibody, rabbit anti-mouse biotinylated (Dako, diluted 1:1000 with blocking buffer, as detailed in Appendix I) was subsequently added. The membrane was incubated for an hour at room temperature on the rotary shaker and again washed as previously stated. Finally, the tertiary reagent, streptavadin alkaline phosphatase (Dako, diluted 1:1000 with blocking buffer, as detailed in Appendix I) was added and incubated at room temperature for 1hr on the rotary shaker. The membrane was then washed for a total of 25mins with 4 changes of tTBS with shaking.

Once the membrane was washed thoroughly, the presence of the protein of interest was visualised with an alkaline phosphatase III substrate kit (Vector Laboratories, SK5300) according to the kit instructions. Briefly this involved incubating the membrane in a developing solution for ~15mins. Negative controls were also carried out where antibodies were emitted. Densitometric analysis was carried out as described in section 2.9.4, however a normal light source was used instead of UV.

2.7.3 Silver Staining

Examination of matrix breakdown was performed using Silver Staining of SDS-PAGE gels in order to optimally detect protein-banding patterns (especially collagens). The ProteoSilver Silver Stain Kit (Sigma) was used, with kit solutions prepared as per kit instructions. The assay was carried out using a plastic tray on an orbital shaker, wearing gloves at all times.

Briefly, after standard electrophoresis an SDS-PAGE gel was placed into a clean plastic tray with 100ml Fixing solution (kit) and shaken at room temperature for 20mins. The fixing solution was then decanted and the gel washed for 10mins in 30% ethanol. This was removed and the gel washed for 10mins in 200mls of dH_2O. Following this, the water was removed and the gel incubated in 100mls of the sensitizer solution (kit) for 10mins. The gel was then washed 2 x in 100mls dH_2O, each for 10mins. Subsequently the gel was equilibrated in 100mls of the Silver solution (kit) for 10mins.

The Silver solution was then decanted and the gel washed in 200mls of dH_2O for 1min (any longer will decrease sensitivity). The gel was then developed in 100mls of

the developing solution (kit) for 3-7mins, until desired level of staining is reached. In order to stop the development, ProteoSilver Stop solution (kit) was added. The stained gels were then photographed using a UVP camera-computer system (Epi Chemi II Darkroom), before carrying out densitometry analysis (see section 2.9.4).

2.7.4 Zymography

2.7.4.1 Zymography Samples

Zymography was carried out to assess the amount of MMP activity in conditioned culture medium. Cells were cultured in 3-dimensional anchored and contractile collagen matrices for 7-days prior to assessing the MMP activity present in the conditioned culture media. As the active protein levels secreted from cells are relative to the specific effects of collagen gel culture, samples were standardised by seeding equal cell numbers into collagen gels at the beginning of the collagen gel culture period. Therefore conditioned media was from equivalent cell numbers in each gel. In addition, the media used for experimentation was low in nutrients so as to maintain cells quiescent (Appendix XII and Figure 3.2, Chapter 3).

For each experimental variable for MMP activity assessment 500µl of conditioned media (conditioned for 3-days, and collected from triplicate gels) was added to 500µl 2 x zymography sample buffer (180mM Tris-HCL pH 6.8, 60% SDS, 30% Glycerol, 0.13% Bromophenol blue) and stored in 1.5ml microfuge tubes at -20°C until analysis.

2.7.4.2 Zymography Gels

10% SDS separating gels were cast containing 1mg/ml gelatin (Sigma, Type A from porcine skin) with a 5% SDS stacking gel set on top. Gels were assembled into a running tank with 1 x running buffer, 25µl of each sample was then loaded into the gel. The entire gel tank was placed in an icebox and left to run for 2.5hrs at 300V, 25mA/gel.

The gels were then placed in 100ml renaturation buffer (2.5% Triton X-100, 1µM $ZnCl_2$ in dH_2O) per gel and incubated at room temperature on an orbital shaker for

2hrs. This buffer was then replaced with excess developing buffer (50mM Tris base, 200mM NaCl, 5mM $CaCl_2$ (anhydrous), 1μM $ZnCl_2$, in dH_2O). The gels were incubated at room temperature with shaking overnight.

The gels were stained with Coomasie (0.5% Brilliant Blue R-250, 40% methanol, 10% acetic acid in dH_2O) for 1hr, and then destained (40% methanol, 10% acetic acid in dH_2O) by shaking for 1-2hrs with several changes of destain solution. The gels were then placed in a square petri-dish with H_2O and photographed using the UVP camera-computer system (Epi Chemi II Darkroom), before carrying out densitometry analysis (see section 2.9.4).

2.8 Apoptosis Assays

2.8.1 Detection of Apoptosis Related Proteins

Doxorubicin was used to induce apoptosis of cells in monolayer culture in order to assess the activity of apoptosis related proteins.

Apoptosis of fibroblasts was induced using 5ng/ml Doxorubicin (Sigma) treatment. The culture media was removed from 2x 75mm^2 adherent tissue culture flasks and replaced with either 10mls of serum free media (SFM) alone (control) or SFM + 5ng/ml Doxorubicin. Cells were incubated for up to 24hrs. After the appropriate incubation time, cells were lysed and harvested for SDS-PAGE and Western blotting analysis, as in section 2.7. Jurkat cells were used to initially test for the optimum time-point to detect activated apoptotic proteins, see Appendix XI. The antibodies that were used are detailed in Appendix II.

2.8.2 Terminal Deoxynucleotidyl Transferase End-labeling – Apoptosis Staining

Staining of apoptotic cells was carried out using the ApoBrdU Kit (product number 556405) from BD-UK Limited. The methodology was adapted depending on sample type to be assayed as follows.

2.8.2.1 ApoBrdU Staining of Cells within 3-D Matrices

At day-7 of the extracellular matrix gel culture period, gels were fixed in 1% paraformaldehyde overnight at room temperature. The gels were retained within their 6-well plates throughout the staining procedure.

Each gel was washed with 100-200µl of wash buffer (kit component) and drained. 100µl of DNA tagging solution (kit) was then added per gel and incubated at 37°C for 1.5hrs.

The gels were subsequently washed with 200µl of rinse buffer (kit) three times. 100µl of the anti-BrdU antibody solution (made up as per kit instructions) were then added to each gel and incubated in the dark at room temperature for 1hr. After 30mins a further 100µl of propidium iodide/RNAse solution (kit component) were added to each gel and incubated for the remaining 30mins. Gels were finally washed with 200µl of wash buffer (kit) before mounting on a microscope slide with Vectashield (Vector, H-1000). Apoptotic cells were visualised using FITC fluorescence microscopy (Zeiss, microscope). The apoptotic nuclei stained fluorescent green and the nuclei of other cells stained red.

2.8.2.2 ApoBrdU Staining of Cells on Monolayer

$3x10^5$ cells were seeded in NGM onto sterile cover-slips in 6-well tissue culture plates. After 48hrs culture media was refreshed with either: SFM (control), SFM + 10% ethanol or SFM + 1mg/ml Doxorubicin. Cells cultured in SFM alone or with 10% ethanol were cultured for 7hrs before fixing the cells. Cells cultured in Doxorubicin however, were fixed after 24hrs of treatment. Cells were fixed in 1ml of 1% paraformaldehyde solution overnight at room temperature.

Cover-slips were glued, cell-side uppermost, using DPX (BDH) to cover-slides. Once dry, cells were stained as in section 2.8.2.1.

2.8.3 Anoikis

Cells were assessed for their ability to undergo anoikis, a form of apoptosis induction that occurs through the loss of cell adhesion.

Bacterial and tissue culture-grade petri-dishes (Greiner, Bio-one) were used for this assay. Bacterial petri-dishes were coated in a 1% BSA solution, which further prevents cell adhesion to the plastic surface. 3 x 10^5 cells were seeded into 1x tissue culture grade petri-dish (control checking initial viability of cells) and 2 x bacterial grade petri-dishes in serum free media. Cells adhered to tissue culture petri-dishes, but did not adhere to bacterial grade petri-dishes.

After 24hrs of non-adherent culture, cells from 1x bacterial grade plate were transferred into a tissue culture grade plate. After 48hrs the non-adherent cells in the 2nd bacterial grade plate were transferred to a tissue culture grade plate. Cells that were able to attach and spread out in tissue culture plates after a period of non-adherent culture were said to be resistant to anoikis apoptosis cues. The cells that remained rounded-up and floating were said to have undergone anoikis. Death was confirmed using vital staining with propidium iodide (25μg/ml in PBS). Only the cells with porous cell membranes (dead or dying cells) took up the dye and stained red under TRITC fluorescence microscopy.

2.8.4 Apoptosis Induction with Extracellular Matrix Fragments

2.8.4.1 Cell Death Induction with Digested Acellular Collagen Gel Fragments

6-well tissue culture grade plates were coated with collagen type 1 (Sigma, type I rat tail) as follows: the stock collagen (2.5mg/ml in 0.1M acetic acid) was diluted to 10μg/ml in PBS (with Ca and Mg), 200μl of the dilute collagen was added to each well of a 6-well plate, the plate was then incubated at 37°C overnight. Before use the wells were washed 2 x with 100μl PBS.

Cells were seeded onto collagen-coated 6-well plates ($3x10^5$ cells/well) in serum free media (SFM) and cultured for 48hrs before adding experimental solutions.

An appropriate number of acellular collagen gels were prepared as in section 2.3.1.3. and left to set for 24hrs. All the acellular collagen gels were pooled together in a 50ml centrifuge tube and digested with a solution of collagenase D (0.05% Collagenase D (Roche), 0.5% BSA in PBS (+ Ca and Mg)), 1ml per gel. Gels were digested for 30mins in a 37°C shaker. Whilst the collagen gels were being degraded, two VivaSpin

filter (VivaScience, MW cut off 10kDa) 50ml centrifuge tubes were washed twice by adding 20mls PBS (with Ca and Mg) and centrifuging at 14,000xg for 10mins. The VivaSpin tubes were then allowed to dry in the tissue culture hood. 20mls of the digested collagen gel solution was added to one spin tube, to the other collagenase-D solution alone (as a -ve control) was added. The tubes were centrifuged at 14,000xg for 20mins.

The <10kDa centrifugates harvested from the collagenase-D and digested collagen gel solutions were then pipetted into separate fresh 50ml centrifuge tubes and an inhibitor added (Aprotinin 100IU). Prior to adding experimental solutions, cells were washed twice with PBS (without Ca and Mg). 1ml of each test solution was added to tissue culture wells in triplicate. Cells were assessed for cell death by light microscopy at 4hrs, 12hrs, 24hrs and 48hrs. Cells that appeared rounded-up and floating were tested to determine if death had actually occurred via apoptosis using the ApoBrdU kit (see section 2.8.2) or propidium iodide vital staining as in section 2.8.3.

2.8.4.2 Apoptosis Induction with Synthetic RGD-Motif Containing Peptides

Cells were seeded onto collagen coated (as detailed in section 2.8.4.1) cover-slips in 6-well plates in SFM for 48hrs before adding experimental solutions consisting of 1mM RGD-peptide (GRGDNP, Calbiochem) or 1mM RAD peptide (GRADSP, Calbiochem) in SFM.

For each cell line $3x10^5$ cells were seeded into triplicate wells for each treatment group: 24hr active peptide treated cells (RGD-peptides), 48hr active peptide treated cells (RGD-peptides), 24hr inactive peptide treated cells (RAD-peptide), 48hr inactive peptide treated cells (RAD-peptides) and 48hr non-treated control cells (serum free media). 1ml of each treatment media was added to each well. At 24hrs and 48hrs appropriate cover-slips were harvested and fixed in 1% paraformaldehyde solution overnight at room temperature. Cell death was assessed microscopically and any floating cells harvested for analysis. Apoptotic cells were detected on the cover-slips and in the harvested floating cell fractions using the ApoBrdU kit (see section 2.8.2).

2.8.4.3 Induction of Apoptosis with Conditioned Homogenised Collagen Gels

Cells were cultured in anchored or contractile collagen matrices for 7-days in the appropriate culture medium. See section 2.3 for collagen gel culture methods. Triplicate matrices of the same treatment group (containing cells) were then pooled together and homogenised with a mortar and pestle in PBS (with Ca and Mg). Once sufficiently homogenised, a protease inhibitor was added (aprotinin 100IU) to prevent non-specific cell death. 1ml of this solution was then added to monolayer-cultured cells that had been set up as stated in 2.8.4.1. After 24hrs cell death was assessed microscopically and using Trypan Blue viable cell staining (section 2.2.3).

2.9 RT-PCR Methods

Ingredients for all solutions and chemicals used are detailed in Appendix III-V. Primer sequences used are detailed in Appendix VI and RNA sample yield is detailed in Appendix VII. Typical PCR gel results are presented in Appendix VIII and IX.

2.9.1 Harvesting RNA from Cells in Collagen Gels

RNA was harvested from collagen gels at the end of the 7-day gel culture period. Triplicate collagen gels from the same treatment group were pooled into 50ml centrifuge tubes. To each tube 3mls of GT lysis buffer (Appendix III) was added. The gels were left for 3-5-days to dissolve in the buffer at 4°C. The contents of the tubes were then mixed thoroughly by vortexing to ensure cells were lysed.

The mixture was then divided into 0.5ml aliquots in sterile microfuge tubes. In a fume cupboard, 500µl of water saturated phenol, 100µl of (24:1) chloroform:isoamylalcohol ($CHCl_3$), 50µl 2M sodium acetate was added to each aliquot. This mixture was vortexed and incubated on ice for 15mins. These aliquots were then spun in a pre-cooled centrifuge (4°C) at 13,000xg for 15mins. After centrifugation, the top phase (aqueous, containing the RNA) was removed by sterile pipetting into a fresh microfuge tube, taking care to avoid the DNA, which lies at the phase interface. The bottom phase (protein) was discarded. An equal volume of isopropanol (approx 500-600µl) was added to the RNA aliquots, and mixed thoroughly using a vortex. The tubes were then stored at -20°C overnight before proceeding to the next stage.

After removal from the freezer the microfuge tubes were spun in a pre-cooled centrifuge for 30mins at 13,000xg. The supernatant was gently decanted and the residual pellet gently washed with 300µl of ice-cold 70% ethanol. The residue was removed with a fine tip. The RNA pellets were then air-dried overnight at room temperature in a class II tissue culture hood. Once dried, each pellet was dissolved in 20-40µl of DEPC (diethylpyrocarbonate-treated) water (Appendix III) by repetitive pipetting, until the viscosity increased. The RNA aliquots were then incubated at 65°C for 5mins and then either used immediately for preparation of cDNA or stored at -80°C until use.

2.9.1.1Determination of RNA Yield and Quality

A 1µl aliquot of the extracted RNA was diluted with 999µl of DEPC-treated water and its absorbance determined spectrophotometrically. The spectrophotometer (ComSpec M330) was zeroed by making a reference against DEPC-water alone, and absorbance readings were taken at 260 nm (A_{260}) and at 280 nm (A_{280}). 1 absorbance unit (A_{260}) equals 40µg of single stranded RNA per ml (Sambrook *et al* 1989).

The purity of the extracted RNA was estimated by comparing the ratio of absorbancies A_{260} : A_{280}. Pure RNA gives a ratio of 2:0, however sample ratios between 1.7 and 2.1 are deemed acceptable (Sambrook *et al* 1989). The concentration and subsequently the total RNA yield was calculated from the A_{260} reading : -

mRNA concentration (µg/µl) = [A_{260} × 40 × dilution factor (1000)]/1000 (µl)

mRNA yield (µg) = mRNA concentration × total volume of pooled RNA

2.9.1.2 Determination of RNA Integrity

The integrity of the RNA was checked using agarose gel electrophoresis, by checking that the ratio of 28S to 18S eukaryotic ribosomal RNAs was approximately 2:1 indicating that no gross degradation of the RNA had occurred.

The gel equipment was assembled (Gibco BRL Electrophoresis power source (model 250Ex), plus BioRad gel tank) and a 0.8% agarose gel solution was prepared (0.8g agarose, with 100ml 1×TAE). The gel mixture was microwaved (Proline microchef 950W, category E, at power level 6) for 2-4mins, stirring frequently, until fully

dissolved, and then left to cool to approximately 60°C. 5µl of ethidium bromide (Invitrogen Cat 15585-011, 10mg/ml) was added to the solution and mixed well. The gel was then poured into the mould and an 8-well comb added. The gel was then allowed to set for 30mins and placed into an electrophoresis tank (BioRad Sub Cell GT mini) filled with 1×TAE buffer. 20 µl of each RNA sample was mixed with 2µl of loading buffer (15% Ficoll 400 in dH_2O, 0.25% xylene cyanol FF, 0.25% bromophenol blue, 30% glycerol in dH_2O) and loaded into the gel well. A 1Kb DNA ladder was loaded into a separate well as a marker (Invitrogen Cat 15615-016). The gel was run for 20mins at 100V. Analysis was performed under an ultraviolet light source (UVP camera-computer system, Epi Chemi II Darkroom). Photographs were taken and analysed using Lab Works version 4.0 (UVP, Bioimaging Systems).

2.9.2 cDNA Preparation

All reagents were thawed on ice except for the reverse transcriptase (RT), which was kept at -20°C until immediately before use. The RNA from the stock solutions was first diluted in DEPC-water to achieve a concentration of 5µg RNA in 8µl in fresh sterile PCR grade microfuge tubes. These samples were then heated at 65°C (heating block) for 10mins and placed on ice for a further 5mins.

In a separate tube the RT working mix was prepared (4µl 5× RT buffer, 2µl 0.1M DTT, 1µl oligo-dT primer, 1µl DEPC-water, 2µl 10mM dNTP, per RNA sample). 10 µl of the working mix was added to each sample, along with 1µl of RNA guard (an RNAse inhibitor) and 1µl of RT (200IU/ml). The samples were then vortexed, pulse centrifuged and incubated at 37°C for 1hr in a water bath. The reaction was terminated by heating samples to 75°C (heating block) for 10mins. cDNA samples were then centrifuged at 13,000xg for 1min and stored at -80°C or used immediately for PCR.

2.9.3 The Polymerase Chain Reaction (PCR)

PCR utilizes two primers (short, single stranded DNAs) complementary to the ends of each specific sequence to be amplified. The extension of each primer creates a DNA strand including the sequence complementary to the opposite primer. PCR is a cyclical process in which the DNA is denatured to become single stranded, the

primers are allowed to anneal with each single strand and then they are extended by utilising nucleotides. A DNA molecule is synthesised which is complementary to the template strands. With each PCR cycle there is an exponential amplification of the specific DNA sequence.

2.9.3.1 Oligonucleotide Primers

A consensus of opinions has provided a set of guidelines for the design and optimal use of a primer pair (Innis *et al,* 1990; Taylor, 1992; Kocher *et al.*, 1994; Old & Primrose, 1994). These guidelines suggest that primers should range from 15-30 bases in length, base composition should be 50-60% guanine and cytosine, long runs with more than three or four of the same base should be avoided. In addition, primers should not have hairpin loops or contain sequences that are complementary to each other, therefore avoiding primer-dimer formation. The primer melting temperature (Tm) should be 55-80°C, with both primers having close Tm.

Specific human oligonucleotide primers were chosen to amplify the various variable portions for MMP-1, 2, 3, 13 and TIMP-1, 2, 3 and 4. Primers spanning two intron-coding regions and one exon region were used. In addition, primers were checked for specificity and cross-reactivity using BLAST. GAPDH was the housekeeping gene used as an internal cellular control. All primers were ordered through and synthesised commercially by MWG Biotech. The internal control is required to compare the changes in mRNA transcripts of the gene of interest. It was important that the mRNA levels of the housekeeping gene did not change with the alterations in tension given to the gel. It has previously been reported that GAPDH is *mechano-insensitive* (Mudera *et al* 2000; Spofford *et al* 2003; Cheema *et al* 2003; Jemiolo *et al* 2004; Tan *et al* 2004) hence it was chosen as a housekeeping gene for these experiments. Controls were carried out to assess the affect of matrix tension (anchored and contractile collagen matrix culture) on housekeeping gene expression; results determined no difference.

2.9.3.2 Setting up the PCR Reaction

All reagents were stored on ice, except DNA polymerase, which was kept at -20°C until just before use. A negative control (DEPC-water only) was used for each PCR

reaction to control for contamination of stock solutions. 2µl of each cDNA sample was aliquoted into a fresh 0.5ml PCR grade microfuge tube. In a separate tube the PCR working mix was prepared (2µl 10×PCR buffer, 2µl 2mM dNTPs, 2µl of each forward and reverse housekeeping primers (10pM, GAPDH), 2µl of each forward and reverse test primers (10pM), 1µl DMSO, 4.75µl DEPC-water and 0.25µl 5U/ml DNA polymerase). Full details of each primer sequence used are given in Appendix VI. This 18µl mix was added to each aliquot of cDNA and mixed. The PCR reaction mixture was overlaid with 2 drops of mineral oil and placed into a PCR machine (Techgene-Techne, Jenkons PLS). The PCR reaction program consisted of 30 cycles (Appendix V).

2.9.3.3 PCR Gels

2% agarose gels were made by dissolving 2g agarose in 100ml of 1×TAE buffer and heating in a microwave for 2-4mins stirring frequently as discussed previously in section 2.9.1.2. The gel solution was then allowed to cool to approximately 60°C before adding 5µl of ethidium bromide and casting the gel. The gel was left to set for 30mins. At this stage the gel was gently inserted into an electrophoresis tank filled with 1×TAE buffer. 20µl of each PCR sample was mixed with 2µl of blue dye (15% Ficoll 400 in dH_2O, 0.25% xylene cyanol FF, 0.25% bromophenol blue, 30% glycerol in dH_2O). Each sample was then loaded into the gel alongside a well containing 6µl of a 1,2,3, DNA ladder (Invitrogen-Cat 15613-029). The gel was run at 100V for 20mins, or until the dye front had migrated down to two thirds of the gel. The gel was then viewed and photographed using the UVP camera-computer system and analysed using Lab Works version 4.0 (UVP, Bioimaging Systems) (Typical results are presented in Appendix VIII and IX).

2.9.4 Image Analysis

Gel analysis was performed using the Lab Works Image Acquisition and Analysis Software system (UVP Laboratory Products). The gels were scanned within a UVP Epi Chemi II Darkroom using an ultraviolet source and a digital camera. Bands were identified using the computer software above, and intensities quantified. The genes of interest were assessed on 8 separate cell-strains of each tissue type. Each PCR reaction was also performed three times. The mean intensities for GAPDH were quantified and then compared to mean band intensities for the test genes. This

resulted in relative band intensities. Results represent the level of expression of the gene of interest as a ratio to that of the house-keeping gene.

2.10 Statistical Analysis of Data

Data was analysed using students T-test analysis, paired T-test analysis or two-way repeated measures ANOVA as appropriate, with $p<0.05$ being judged as statistically significant. All statistical analysis was performed using SigmaStat (Jandel) software.

Section A

Chapter 3

Examination of the Ability of Scar Fibroblasts to Undergo Apoptosis

3.1 Introduction

As stated previously in chapter 1, during normal wound healing, a steady-state exists between cell proliferation and apoptosis (Desmouliere *et al.,* 1995), leading to the controlled cessation of the wound repair process, thus producing an acellular and flat scar. Disruption of this critical balance results in wound healing pathogenesis, either failure of wound healing as seen in chronic wounds or excessive healing (fibrosis) as seen in keloid scars. As previously described in section 1.5, a prolonged presence and activation of fibroblasts is commonly found in active keloid scars. This phenomenon could theoretically be caused by unremitting induction of these cells or their failure to undergo apoptosis (Appleton *et al.,* 1996). The prolonged presence of active fibroblasts in keloid scars could feasibly be sufficient to cause this fibrotic pathology.

Published research to date on this hypothesis, attempting to investigate the apoptotic activity or capability of keloids, has so far only presented insufficient or conflicting evidence, as described in section 1.7. This chapter concentrates on investigating whether keloid fibroblasts are able to respond to cues of apoptosis, specifically those potentially associated with wound healing, and to further characterise any defects seen.

3.1.1 Wound Healing Apoptosis Cues

The main problem to date with this line of investigation has been that little is known about the physiological cues of apoptosis that take place during wound healing progression. This fact together with clear evidence that the mechanism of apoptosis alters depending on induction methods, cell type, and local environment amongst other factors makes any attempt to extrapolate most of the published studies to the healing wound unsound.

Thus, the stimulus that leads to apoptosis induction during wound healing remains unclear. Many different gene products are known to affect apoptosis; a number of which have been shown to have a role in the apoptosis of fibroblasts specifically, although a direct link with wound healing remains to be established. Amongst these the c-myc protein is also implicated in co-transformation, auto-regulation and inhibition of differentiation, this suggests that its apoptotic function may relate to

these other effects (Evan *et al.*, 1992). Also, interleukin-1β-converting enzyme (ICE) has been implicated in apoptosis induction, through its over-expression in rats (Miura *et al.*, 1993). In addition it has been shown that Bcl-2, a mammalian oncogene, is capable of blocking apoptosis (Miura *et al.*, 1993). Nevertheless, these factors have no known relevance to the healing wound.

A possible mechanism for apoptosis induction could also be via direct action and /or withdrawal of cytokines or growth factors (Laster *et al.*, 1988; Robaye *et al.*, 1991; Oberhammer *et al.*, 1993; Moulton, 1994). Several factors have been shown to increase the rate of wound healing, including PDGF, TGF-β, TGF-α and TNF (Pierce *et al.*, 1988; Mustoe *et al.*, 1987; Schultz *et al.*, 1987; Mooney *et al.*, 1990; Beck *et al.*, 1993). A possible explanation for the death of at least a subpopulation of cells could be that these are growth factor dependant. Alternatively, factors selectively causing the death of fibroblastic cells could be liberated after epithelialisation has been completed (Desmouliere *et al.*, 1995). To date there is still no specific mechanism of apoptosis induction published.

The only clue to the potential mechanism of apoptosis in a wound healing context is from the work of both Fluck *et al* (1998) and Grinnell *et al* (1999), which indicates a potential role for fibrillar collagen. Both research groups found that when human dermal fibroblasts were allowed to remodel and contract a 3-D collagen type I matrix they underwent apoptosis. This *in vitro* model has been suggested to mimic the events that take place during wound healing (Bell *et al.*, 1979; Fluck *et al.*, 1998; Grinnell *et al.*, 1999), and is thus a more physiologically relevant method of inducing apoptosis by which to test for defects in keloid scar-derived fibroblasts than those tested previously (section 1.7).

3.1.2 The In Vitro Model of Wound Healing

Several *in vitro* models have been used to assess the contraction of tissue by cells, and the contraction of cells themselves using fibroblasts cultured within 3-D matrices (Ehrman and Grey, 1956; Elsdale and Bard, 1972; Delvoye *et al.*, 1991; Tomasek *et al.*, 1992; Kolodney and Wysolmerski, 1992; Eastwood *et al.*, 1994, 1996). Fibroblasts cultured in collagen matrices have been found to acquire tissue-like

phenotypic characteristics not typically observed when cultured in monolayer (Elsdale and Bard, 1972). Typically, fibroblasts cultured at low density in monolayer exhibit one or more large flattened pseudopodia, with membrane ruffling, however when cultured within an anchored 3-D collagen matrix the cells adopt a mainly bipolar spindle form, where several fine pseudopodia extend from two opposing ends of the cell (Elsdale and Bard, 1972). When matrices are un-tethered and allowed to float in the culture medium, cell motility (cells pulling on the collagen fibrils as they move towards each other) results in the collapse of the matrix into a dense, opaque body less then one-tenth of the original size (Elsdale and Bard, 1972). The same is observed with fibrin matrices (Niewiarowski and Goldstein, 1973). The fibrin culture matrix is thought to provide an environment similar to that of the early phases of wound repair, whereas a collagen matrix environment mimics that of the later stages of wound repair. Subsequently the use of collagen-matrix reorganisation as an *in vitro* model of wound contraction has been studied (Bell *et al.,* 1979). The three main variations of the *in vitro* collagen matrix contraction model used by research groups are illustrated in Figure 3.1

The three models in Figure 3.1 vary distinctly in their mechanical features. In I, tension is distributed isotropically (tension is the same irrespective of direction), in II, tension is distributed anisotropically (tension is different in different directions) and in III, mechanical stress develops during the period the matrix is maintained anchored and then stress dissipates after the matrix is released. When comparing contraction of floating matrices vs the anchored collagen matrix, the major difference is that contraction of floating matrices results in a mechanically relaxed tissue, whereas the contraction of an anchored matrix results in a stressed tissue (Grinnell, 1994).

The work carried out in this thesis involves investigating the effects of both anchored matrix contraction (II); which is thought to closely mimic the fibro-proliferative, early granulation tissue remodelling phase of wound healing, and the effects of stress-relaxed matrix contraction (III); which is thought to mimic the late granulation tissue remodelling phase of wound healing.

An adaptation of this model is seen in Fluck *et al's* (1998) work, where the 3-D collagen matrix differs slightly from that of Grinnell (1994); in that the matrices were

not droplets of collagen in the centre of tissue culture wells, but rather gel discs, which were attached to the sides of the tissue culture well, as well as the base. It is possible that the distribution of tension within tethered gels may differ slightly from the gel droplets, nevertheless both research groups found that apoptosis was induced on the release and subsequent contraction of a pre-stressed collagen matrix (analogous to model III, Figure 3.1 - Fluck *et al.*, 1998; Grinnell *et al.*, 1999). This model of wound contraction has thus been used as a method of studying the apoptosis that occurs during wound healing and its pathologies.

MODEL	**I) Free floating matrix contraction**	**II) Anchored matrix contraction**	**III) Stress-relaxed matrix contraction**
MEASUREMENT	Reduction in diameter	Reduction in height	Reduction in diameter after release from anchorage
MECHANICS	Isotropic tension distribution. Mechanically relaxed tissue	Anisotropic tension distribution. Stressed tissue	Initially mechanically stressed, then mechanically relaxed

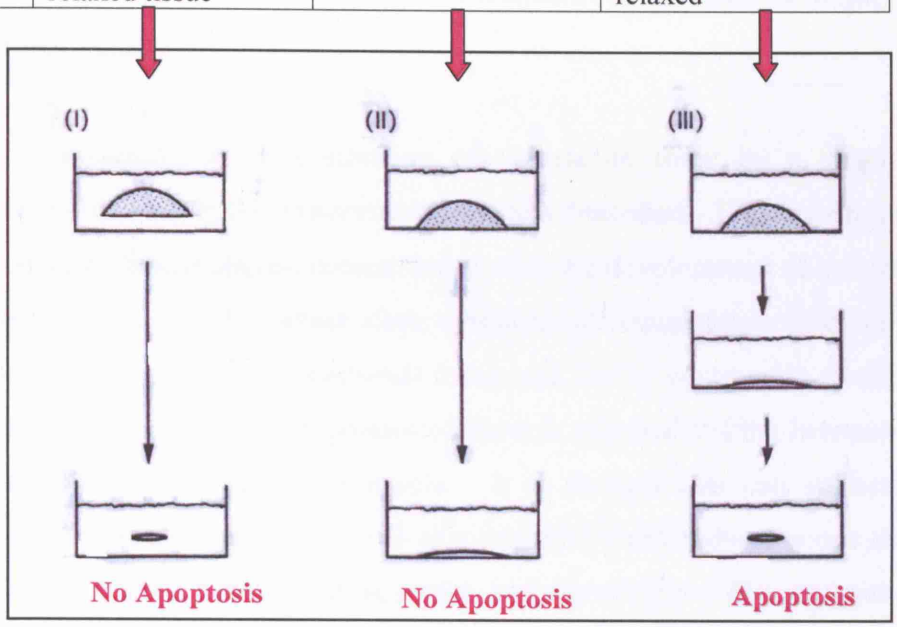


Figure 3.1 Illustration of the three common variations of the collagen matrix contraction model. I) floating matrix contraction, II) anchored matrix contraction and III) stress-relaxed contraction (adapted from Grinnell, 1994).

Since collagen contraction is the primary instigator of apoptosis in the above model it is important to understand its potential mechanisms.

3.1.3 The Mechanisms of Collagen Matrix Contraction

The mechanism of free-floating and anchored matrix contraction is postulated to occur primarily as a consequence of motile activity by cells migrating through the matrix (Harris *et al.*, 1981). This process is called 'tractional remodelling' to distinguish it from smooth-muscle-like contraction. More specifically, contraction occurs as fibroblasts spread and elongate, not as already elongated cells retract their extensions (Grinnell and Lamke, 1984). Cells act to reorganise the nearby collagen fibrils and subsequently the rest of the matrix as tractional forces are transmitted throughout the network of collagen matrix fibrils (Guidry and Grinnell, 1987). In addition, tethered gels develop an isometric force as the resident cells contract against the fixed points. On release of anchored matrices (stress-relaxed), a rapid contraction occurs that is proportional to the isometric force generated by the cells (Grinnell, 2003). This is followed by the contraction of smooth muscle actin expressing cells, along-side the continual process of tractional remodeling, whereby cells continue to realign themselves, remodelling their matrix environment to produce a highly organised and compact matrix.

Several different components of the generation of contractile force by a single fibroblast have been defined using the experimental models described. The process is thought to involve several linked stages, commencing with the development of cell to matrix contacts, recruitment of cell contact sites, cytoskeletal organization with cell spreading, the generation of cell-matrix tractional force, and finally contraction (Sethi *et al.*, 2002). Possibly one of the most important of these is physical linking between the cell cytoskeleton and the extracellular matrix. It is thought that cell surface receptors known as integrins, play a vital role in this process by attachment to one or more matrix ligands, including collagen, fibronectin, and vitronectin. The integrins are a family of extracellular matrix receptors present on a cell surface which act by linking extracellular macromolecules with the cytoskeleton and promoting cell attachment, migration and shape changes (Burridge *et al.*, 1988; Clyman *et al.*, 1990). Cell attachment to collagen within collagen gels has been shown to be fibronectin dependant, with the contractile force being specifically dependant on the $\alpha 2\beta 1$ integrin-matrix interaction (Klein *et al.*, 1991; Schiro *et al.*, 1991; Langholz *et al.*, 1995; Xu *et al.*, 1998), but independent of other integrin interactions such as that of the classic fibronectin receptor, $\alpha 5\beta 1$ (Tomasek and Akiyama, 1992). Recent work

has indicated that there may be a short sequential cascade of integrin utilisation in the early phases of fibroblast contraction commencing with fibronectin receptors and ending with the collagen integrin subcomponent (Sethi *et al.,* 2002).

Tensile forces generated are dependant on an intact actin cytoskeleton (Bell *et al.*, 1979; Bellows *et al.*, 1982; Guidry and Grinnell, 1985). Forces applied through the membrane-bound integrins are transduced via focal adhesion complexes into intracellular signals that mediate the redistribution of cytoskeletal proteins, and lead to down-stream signalling and gene transcription of cytoskeletal genes such as actin, filamin and vinculin (D'Addario *et al.,* 2001). Part of the contractile force generated by the fibroblast is due to stored energy within the cell's actin-myosin motor elements of the microfilaments (Brown *et al.*, 1996). This force is proposed to be held as a compressive load that maintains cell shape. In addition, microtubules within the cell act as an intracellular framework, which counteracts the pulling force of the microfilaments and maintains the cell morphology (Brown *et al.,* 1996).

Collagen matrix contraction has been found to require serum (Tomasek *et al.*, 1992), which indicates cell contractility can be controlled by extracellular factors. TGF-β1 for instance stimulates contraction of collagen matrices and promotes fibroblast to myofibroblast differentiation. PDGF acts as a stimulator of matrix contraction, although by a separate mechanism to that of TGF-β1 (Grinnell, 1994). Factors that inhibit gel contraction include; FGF and IFN-γ (Grinnell, 1994). To date the mechanisms involved in these induction/inhibition pathways are still unknown.

3.1.4 Morphological Features of Fibroblasts within 3-D Matrices

On contraction of a floating matrix, cells acquire morphological and proliferative features resembling those of resting dermis, whereas cells within anchored matrices become stressed and the tissue resembles granulation tissue. Specifically, in floating collagen matrices, fibroblasts develop a quiescent and stellate morphology, with long spindle-like dendritic processes and a very dense cell body (Bell *et al.*, 1979; Bellows *et al.*, 1981; Grinnell, 1994), in contrast to cells within anchored matrices, which become bipolar with strong, prominent cell extensions and elongate along lines of tension (Stopak and Harris, 1982; Bellows *et al.*, 1982). Cells within anchored,

stressed matrices also develop prominent stress fibres, such as α-SMA and fibronexus junctions resembling myofibroblasts (Farsi and Aubin, 1984; Mochitate *et al.*, 1991; Tomasek *et al.*, 1992). Whereas the myofibroblast phenotype is relatively absent in floating matrices that have not been pre-stressed (Grinnell, 1994).

3.1.5 Metabolic Features of Fibroblasts within 3-D Matrices

Previous research has found that fibroblasts cultured within anchored, stressed fibrin matrices continue to proliferate, and synthesise collagens and proteins. On stress-relaxation however, cells stop dividing and although collagen and protein synthesis continues, it is at a significantly lower rate and the cells act to contract the matrix through residual tension (Gillery *et al.*, 1989; Tuan *et al.*, 1996a). As with fibrin matrices, fibroblasts cultured within anchored, stressed collagen matrices synthesise extracellular matrix proteins and proliferate (Nishiyama *et al.*, 1989; Nakagawa *et al.*, 1989a, 1989b). In addition, cells utilise the Rac kinase pathway, which regulates the formation of lamellipodia protrusions and membrane ruffles (Hall, 2005). Rac is part of the Rho family of small GTPases, each of which control signal transduction pathways linking membrane receptors to the assembly and disassembly of the actin cytoskeleton and of associated integrin adhesion complexes (Hall, 2005). During collagen matrix stress-relaxation there is a down-regulation of the ERK pathway, cells utilise lysophosphatidic acid (LPA), $G_{\alpha 12/13}$ and RhoA and become quiescent (Grinnell, 2000). Rho has been found to regulate stress fibre and focal adhesion assembly (Hall, 2005). Specifically cells have been shown to reduce DNA synthesis (Sarber *et al.*, 1981; Nishiyama *et al.*, 1989; Nakagawa *et al.*, 1989a, 1989b), arrest in G_0 (Kono *et al.*, 1990) where cells begin to down-regulate their biosynthetic activity (Nakagawa *et al.*, 1989a, 1989b) and a subpopulation undergoes apoptosis (Fluck *et al.*, 1998; Grinnell *et al.*, 1999).

The low proliferative capacity of fibroblasts in floating matrices appears to reflect the cells decreased responsiveness to growth factors (Nakagawa *et al.*, 1989a). Indeed, PDGF receptors on fibroblasts in floating collagen matrices lose their capacity to autophosphorylate in response to PDGF (Lin and Grinnell, 1993). In addition to changes in cell proliferation, fibroblasts in floating collagen matrices show decreased collagen biosynthesis (Iwig *et al.*, 1981; Mochitate *et al.*, 1991), whereas there is a

marked increase in the release of collagenase compared with cells in anchored matrices (Grinnell, 1994).

3.1.6 Rationale for the Choice of Experimental Model Used in this Chapter

A matrix model was chosen which closely mimics the transition of fibroproliferative granulation tissue to a remodelled resting tissue (scar), the stress-relaxed contraction model (Figure 3.1. III), reviewed extensively by Carlson and Longaker (2004). Within this model, as well as tractional remodelling, the cells themselves contract by the retraction of their pseudopodia and collapse of actin filament bundles (Mochitate *et al.*, 1992; Tomasek *et al.*, 1992). This signifies the involvement of smooth muscle-like contraction rather than tractional remodelling alone (Grinnell, 1994). Intact stress fibres are required (formed during the period the gel is maintained anchored) and the process is regulated by serum factors (Tomasek *et al.*, 1992). Accompanying stress-relaxation, fibroblasts show transient ectocytosis of annexin-containing vesicles (Lee *et al.*, 1993) and release of cell surface fibronectin (Mochitate *et al.*, 1991). Once mechanical stress is relieved, usually by a combination of matrix contraction and biosynthetic activity, cells switch to a non-proliferative phenotype and begin to down-regulate their activity, even in the continued presence of growth factors (Grinnell, 1994).

This stress-relaxed collagen gel model (hereafter referred to as 'contractile' gels) is thought to provide an environment, which mimics the transition of granulation tissue remodelling (an environment under tension), to that of a resolved scar (an environment with a tension resembling that of resting dermis).

The finding published by Fluck *et al* (1998), describing that normal dermal fibroblasts undergo apoptosis after collagen contraction and therefore potentially to the effects of collagen gel remodelling (mimicking that of a closing wound), directed this study to investigate the effects of this model (simulating wound-healing apoptosis cues) on cells derived from normal scar (control) and keloid scar-derived fibroblasts. The model adaptation described by Fluck *et al* (1998), was judged to be more reproducible than that of Grinnell's (1994) model and so the same gel contraction model was used in this study, as described in section 3.1.2.

3.1.7 Aim

This chapter aims to investigate the ability of keloid scar-derived fibroblasts in comparison to that of normal scar-derived fibroblasts to undergo apoptosis to wound healing apoptosis cues and/or whether keloid scar fibroblasts display a general inability to undergo apoptosis.

3.2 Results

3.2.1 Optimisation of Cell Viability in 3-D Matrix Culture

The effect of collagen matrix contraction was investigated using the 'stressed-relaxed' collagen gel model as described previously in section 3.1.2. As in Fluck *et al* (1998), collagen matrices were maintained anchored for a period of 4-days prior to being released in order to ensure fibroblasts were of a stressed (myofibroblast) phenotype (Grinnell, 2003). The matrices were then either maintained anchored for a further 3-days or loosened from the plastic and allowed to contract over 3-days. The original work by Fluck *et al* (1998) performed experiments in the presence of 10% FCS however; it became clear during the optimisation of this experiment that significant differences in cell proliferation occurred under these conditions. Considering that one major difference in cellular behaviour between anchored versus contractile collagen gels is with respect to cell division (continued versus arrest, respectively), performing experiments under conditions that permit significant proliferation may confuse the quantification of apoptosis results.

Examination of total cell number over time in anchored collagen gels under conditions that permit proliferation (10% serum) compared with conditions that induced cell quiescence (1% serum – see appendix XII) was performed over the time course required for the stressed-relaxed collagen gel experiments (7-days). Figure 3.2 and Table 3.1A shows that when culturing fibroblasts within anchored collagen matrices for a period of 7-days a significant degree of cell proliferation occurs when cells are cultured in DMEM + 10% FCS (10% NGM) irrespective of the tissue source. Of the 3 different tissue sources examined (normal scar, keloid scar and normal dermis), both normal scar and keloid scar fibroblast-seeded matrices underwent a significant increase in cell number by day 5 from that at day-0 (53-62 $x10^4$ ($p=0.016$) and 54-65 $x10^4$ ($p=0.041$) correspondingly) (Table 3.1A). Proliferation was initially

lower in collagen gels seeded with fibroblasts derived from normal dermis, becoming significant by day-6 (p=0.042). This increased proliferation was maintained throughout the experimental time-course; with dermal fibroblasts and keloid scar fibroblasts showing a significant increase in live cell number from day-5 to day-6 (69-74 $x10^4$ (p=0.051) and 65-71 $x10^4$ (p=0.040), respectively) and normal scar and keloid scar from day-5 to day-7 (62-70 $x10^4$ (p=0.045) and 65-75 $x10^4$ (p=0.036), respectively) (Figure 3.2 and Table 3.1A). In contrast there was no significant rise in live cell number (irrespective of tissue-source) from day-0 when fibroblasts were cultured in 1% NGM over the whole time-course of the experiment (Tables 3.1B).

All further experimentation examining the induction of apoptosis was therefore performed in this minimal growth medium (containing 1% FCS), which maintained cells in a quiescent but relatively healthy state in order to remove the added complication of changes in proliferation rates between gel types. In addition, the use of minimal growth medium should also reduce the effects of serum-derived growth factors from inhibiting or masking the specific effects of the different 3-D matrices on apoptosis.

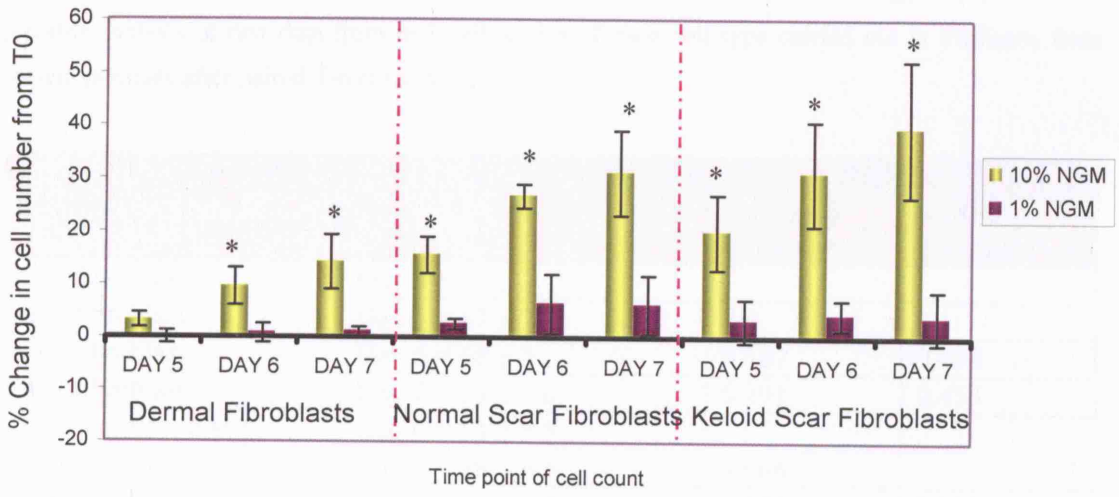


Figure 3.2 Fibroblasts derived from normal dermis, normal scar and keloid scar were seeded into anchored 3-D collagen matrices. Cells were maintained within anchored matrices for a period of 7-days in either DMEM + 10% FCS (10% NGM) or 1% FCS (1% NGM). Live cell counts were performed at day-0, -5, -6 and -7. Results represent a mean of three experiments performed in triplicate with n=3 each of normal dermis, normal scar and keloid scar cell strains. Error bars represent SD. T-test analysis was carried out for each cell type, comparing live cell number at day-0 to that at day-5, -6, and -7. *P=<0.05.

Table 3.1A Statistical analysis of the effects of 10% NGM on the proliferation of fibroblasts derived from normal dermis, normal scar and keloid scar, seeded within anchored collagen matrices. Data represents analysis of raw data from n=3 cell strains of each cell type carried out in triplicate. Data represent p-values after paired T-test analysis (* = p= <0.05).

Table A. Paired T-test analysis of cells cultured in 10% NGM			
Cell Strain	**Mean No. live cells ($x10^4$) ± SD**	**Vs Day-0 P-value**	**Vs Day-5 P-value**
Dermal fibroblast	Day 0 = 67 ± 3		
Dermal fibroblast	Day 5 = 69 ± 2	0.061	
Dermal fibroblast	Day 6 = 74 ± 1	**0.042***	0.051
Dermal fibroblast	Day 7 = 76 ± 1.5	**0.041***	0.142
Normal scar fibroblasts	Day 0 = 53 ± 5.5		
Normal scar fibroblasts	Day 5 = 62 ± 7.9	**0.016***	
Normal scar fibroblasts	Day 6 = 67 ± 5.6	**0.002***	0.130
Normal scar fibroblasts	Day 7 = 70 ± 4	**0.021***	**0.045***
Keloid scar fibroblast	Day 0 = 54 ± 4		
Keloid scar fibroblast	Day 5 = 65 ± 2.5	**0.041***	
Keloid scar fibroblast	Day 6 = 71 ± 2.5	**0.033***	**0.040***
Keloid scar fibroblast	Day 7 = 75 ± 1	**0.034***	**0.036***

Table 3.1B Statistical analysis of the effects of 1% NGM on the proliferation of fibroblasts derived from normal dermis, normal scar and keloid scar, seeded within anchored collagen matrices. Data represents analysis of raw data from n=3 cell strains of each cell type carried out in triplicate. Data represent p-values after paired T-test analysis.

Table B. Paired T-test analysis of cells cultured in 1% NGM			
Cell Strain	**Mean No. live cells ($x10^4$) ± SD**	**Vs Day-0 P-value**	**Vs Day-5 P-value**
Dermal fibroblast	Day 0 = 67 ± 3		
Dermal fibroblast	Day 5 = 67 ± 2	1.0	
Dermal fibroblast	Day 6 = 68 ± 2	0.587	0.434
Dermal fibroblast	Day 7 = 68 ± 3.6	0.191	0.453
Normal scar fibroblasts	Day 0 = 53 ± 5.5		
Normal scar fibroblasts	Day 5 = 58 ± 6.4	0.066	
Normal scar fibroblasts	Day 6 = 56 ± 7.4	0.184	0.706
Normal scar fibroblasts	Day 7 = 57 ± 8	0.191	0.685
Keloid scar fibroblast	Day 0 = 54 ± 4		
Keloid scar fibroblast	Day 5 = 56 ± 5	0.318	
Keloid scar fibroblast	Day 6 = 57 ± 5.5	0.126	0.136
Keloid scar fibroblast	Day 7 = 56 ± 5	0.319	0.403

3.2.2 The Effects of 3-D Matrix Culture on Normal Scar and Keloid Scar Cells

Once the quiescent conditions to culture fibroblasts within 3-D matrices had been determined, the effects of a fibrin matrix environment (mimicking the early phases of wound healing) and a collagenous matrix environment (mimicking the late phases of wound healing) was investigated on normal scar and keloid scar-derived fibroblasts.

Induction of contraction by the fibroblasts appeared similar irrespective of matrix composition or the originating scar type. Figure 3.3 shows representative photographs of the degree of contraction induced by normal scar and keloid scar fibroblasts, when seeded into either collagen or fibrin matrices. The matrices were maintained anchored for a period of 4-days prior to allowing the cells to contract them for 3-days. No obvious difference in the overall degree of contraction induced can be seen between normal scar and keloid scar-derived fibroblasts, or between collagen and fibrin matrices (Figure 3.3). On measuring the degree of contraction, which had taken place in collagen matrices by day-7 of matrix culture, statistical analysis of the gel circumference (as a percentage of the original circumference) revealed no significant difference in the total contraction that was induced by the two scar types or between either matrix (Figure 3.4).

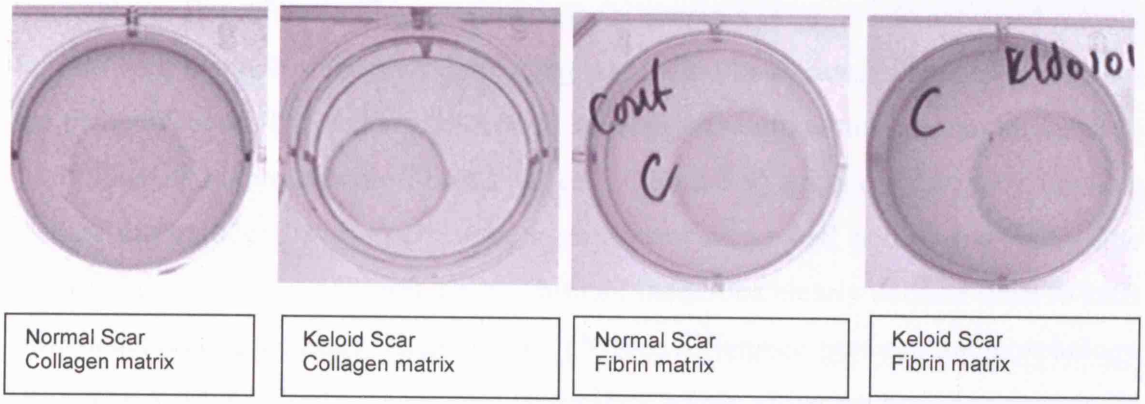


Figure 3.3 Comparative examples of typical gel contraction where fibroblasts derived from normal scar or keloid scar were seeded into collagen or fibrin matrices for 7-days and maintained in 1% NGM. Fibroblasts where allowed to contract the matrix from day-4 of matrix culture for 3-days, prior to this gels where maintained anchored. This result is typical of n=6 cell strains for each scar type.

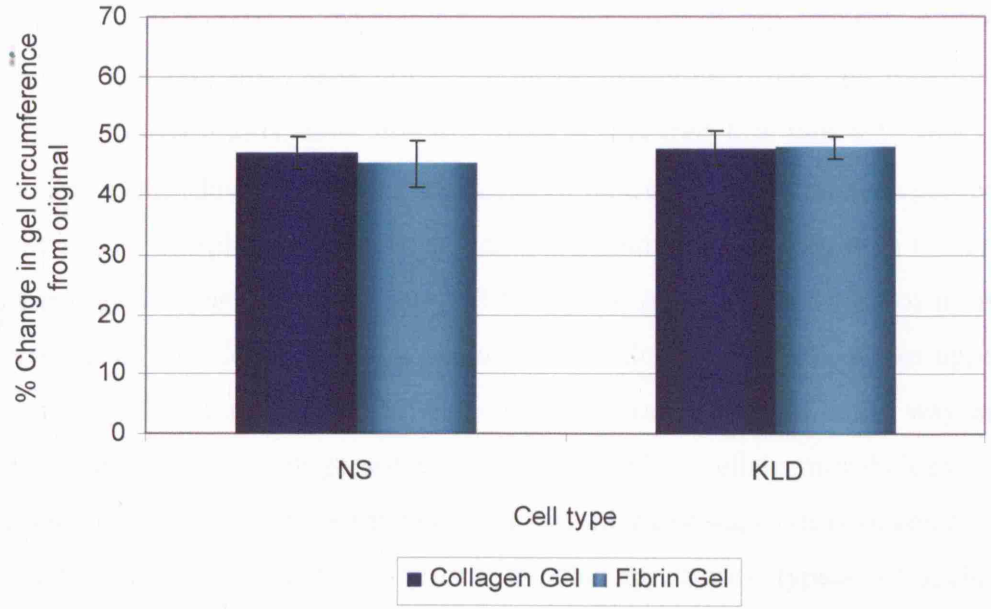


Figure 3.4 Normal scar (NS) or keloid scar (KLD) fibroblasts where embedded into 3-D collagen matrices and cultured in 1% NGM. The matrices where maintained anchored for 4-days before releasing them and allowing them to contract for 3-days. At day-7 of the matrix culture period the circumference of the matrices was measured. Results are representative of the percentage change in matrix circumference from the circumference of the tissue culture well. Results represent a mean of the experiment performed in triplicate with n=6 for each scar type. Error bars represent SD.

In order to examine cell morphology within fibrin and collagen matrices, gels were stained with Toludene blue (Figure 3.5 and 3.6, respectively). Under light microscopy, fibroblasts within anchored fibrin matrices (Figure 3.5) appeared mainly bipolar with a small proportion displaying dendritic-like processes extending beyond the plane of focus. The cells were evenly dispersed throughout the matrix. In contrast, fibroblasts within contractile fibrin matrices (Figure 3.5) generally had very obvious stellate morphology, with exceptionally prominent dense cell bodies and many fine dendritic processes. In addition, cells exhibited numerous clearly defined links to each other's dendritic processes. There was no obvious difference between the morphology of normal scar or keloid scar-derived fibroblasts within either anchored or contractile fibrin gels (Figure 3.5).

Within anchored collagen matrices (Figure 3.6) cells derived from both scar types exhibited indistinguishable morphology and appeared very similar to those in anchored fibrin matrices (Figure 3.5). However, within contractile collagen gels,

although cells generally exhibited a stellate morphology, similar to that seen in contractile fibrin matrices (Figure 3.5), they had few cell-cell contacts, with each cell distinctly separate from each other. On close inspection of the cell processes of the normal scar fibroblasts, they initially (day-5) appeared less taut than that of keloid scar fibroblasts (day-5) in contractile collagen matrices. As contraction proceeded, the difference in morphology of normal scar vs keloid scar fibroblasts in the contractile collagen gels became clearer (Figure 3.6, day-7). A large proportion of normal scar-derived cells rounded up (circles Figure 3.6). Keloid scar cells did not appear to be affected by the later effects of collagen gel contraction in the same way as that of normal scar cells. Although some cells developed a stellate morphology similar to contractile fibrin gels and normal scar cells in the early stages of collagen contraction, a large number of cells maintained the bipolar appearance typical of anchored gels (Figure 3.6). In addition very few cells had rounded-up. In contrast to normal scar cells after contraction the keloid scar cells appeared healthy, stretched and tightly held within the extracellular matrix. The remaining normal scar cells, that had not rounded-up appeared stellate, their processes were not taut within the matrix, but rather loose and non-elastic in appearance (arrows, Figure 3.6).

These results suggest that contractile collagen but not fibrin matrices specifically have detrimental effects on normal scar fibroblasts causing the cells to lose their attachments within the matrix and round-up. This effect is similar to that described for normal dermal fibroblasts by Fluck *et al.,* (1998) and Grinnell *et al.,* (1999). Interestingly, keloid scar cells appear to be unresponsive to this effect of contractile collagen matrices.

In order to establish whether the appearance of rounded up cells within contractile collagen matrices seeded with normal scar cells was indicative of dying cells and more specifically of cells undergoing apoptosis, *in situ* cell staining using terminal deoxynucleotidyl transferase (TdT) end-labelling (ApoBrdU kit from Pharmingen) was carried out. This technique fluorescently labels breaks in cellular DNA, which is characteristic of apoptosis. Typical results are shown in Figure 3.7 and confirm that apoptotic cell death of fibroblasts derived from normal scars was indeed being induced on collagen matrix contraction. No apoptotic nuclei were detected in anchored collagen gels (negative control) seeded with normal scar or keloid scar

fibroblasts after 7-days of matrix culture. In addition, apoptotic nuclei were not evident in contractile collagen matrices seeded with keloid scar-derived cells.

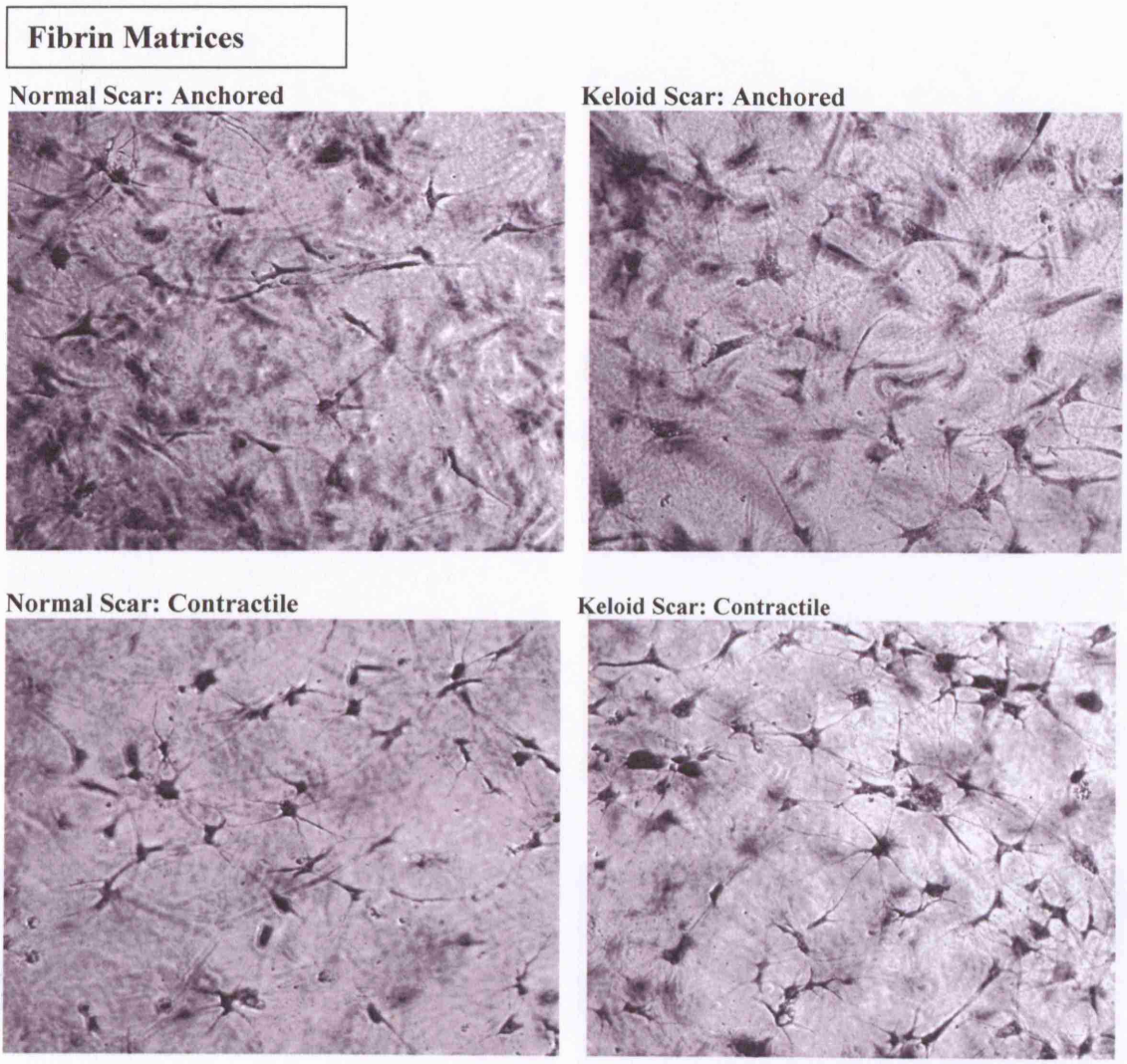
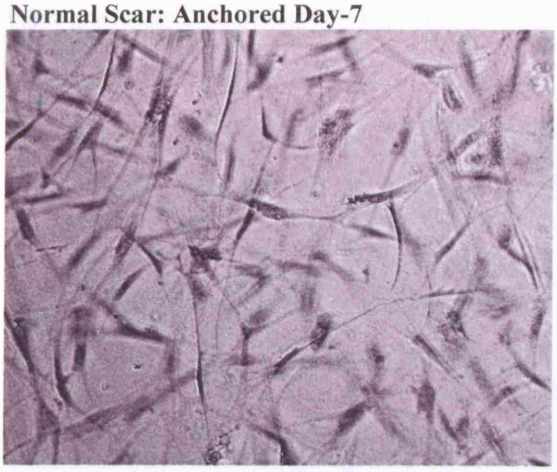


Figure 3.5 Typical Toludene blue staining of anchored and contractile fibrin gel matrices seeded with normal scar or keloid scar fibroblasts. The cells were maintained in 1% NGM, and were cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. These micrographs are representative results of n=5 each of normal scar and keloid scar cell strains performed in triplicate. (x200 Mag)

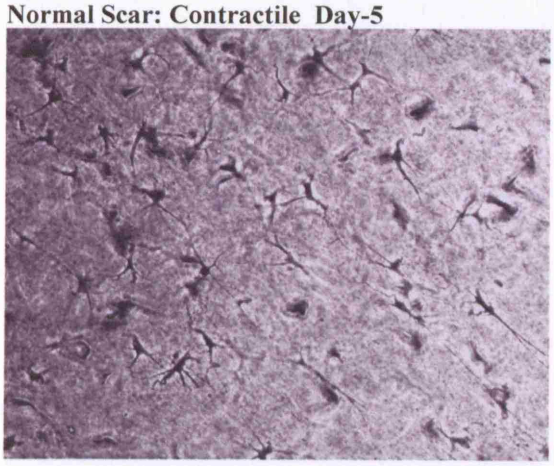
Collagen matrices

Normal Scar: Anchored Day-7

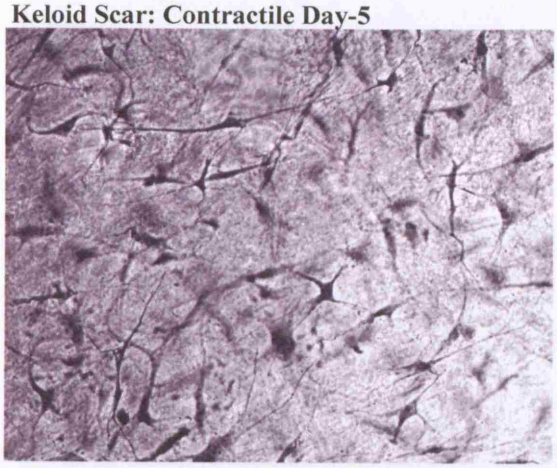


Keloid Scar: Anchored Day-7

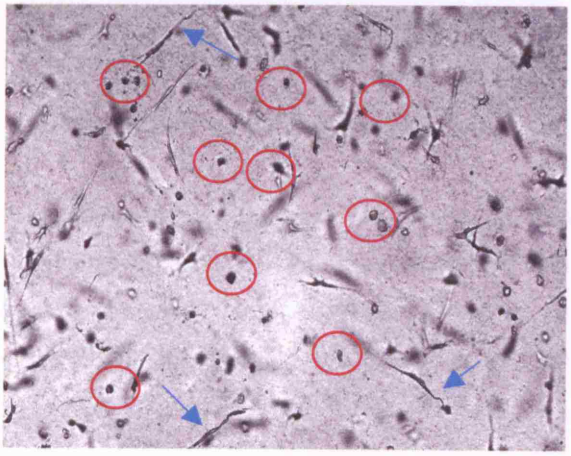
Normal Scar: Contractile Day-5



Keloid Scar: Contractile Day-5



Normal Scar: Contractile Day-7



Keloid Scar: Contractile Day-7

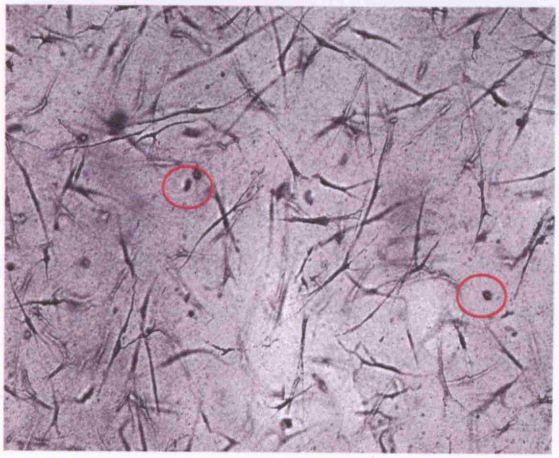


Figure 3.6 Typical Toludene blue staining of anchored and contractile collagen gel matrices, seeded with normal scar or keloid scar fibroblasts. The cells were maintained in 1% NGM and were cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Micrographs show typical cell morphology in anchored matrices at day-7 and contractile matrices at day-5 and day-7. The experiment was carried out with n=5 each of normal scar and keloid scar cell strains in triplicate. (x200 Mag).

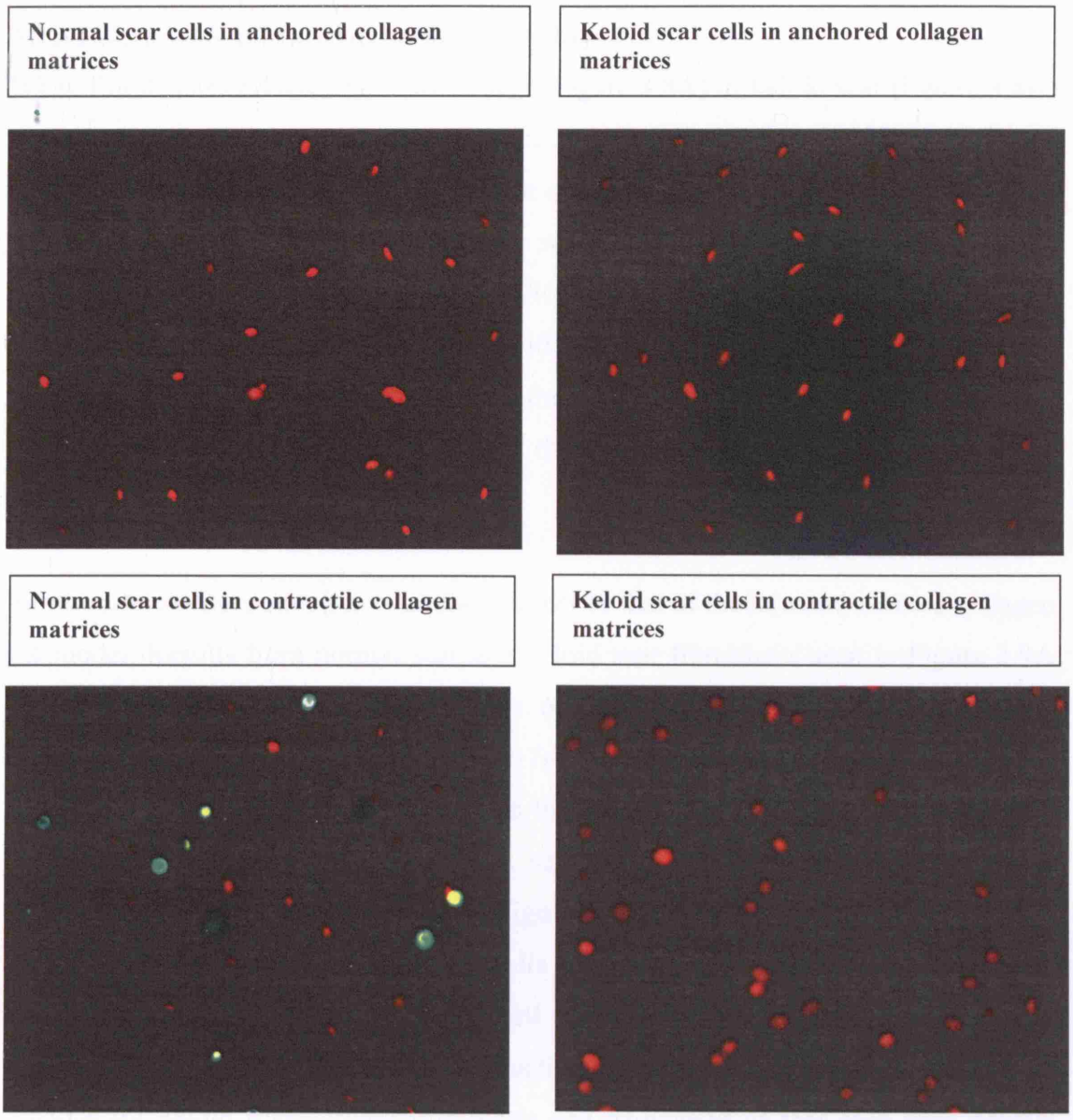


Figure 3.7 Representative terminal deoxynucleotidyl transferase end-labelling of apoptotic nuclei (green) with red (propidium iodide) counterstaining. Staining was carried out on day-7 anchored and contractile collagen matrices seeded with either normal scar or keloid scar cells. Cells were maintained in 1% NGM for the 7-day culture period. These results are representative of n=5 normal scar and keloid scar cell strains in triplicate. (x200 Mag)

These results suggest that like dermal fibroblasts, contraction of a collagen matrix signals the apoptosis of fibroblasts derived from normal scars, but not evidently of those derived from keloid scars. In order to confirm this observation, quantitative comparison of cell death induced by collagen contraction was performed in minimal growth medium (1% FCS) using viable staining.

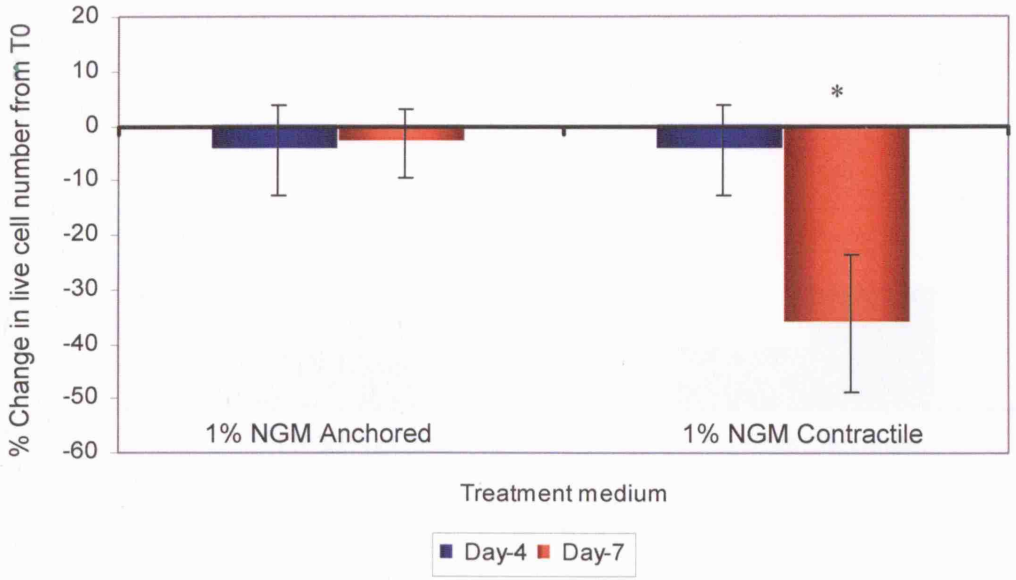
3.2.3 Effects of 3-D Matrices on Cell Viability

When fibroblasts derived from normal scar (Figure 3.8A) or keloid scar (Figure 3.8B) were embedded into collagen matrices, which were maintained anchored (experimental –ve control) for the duration of the experimental time course (7-days), no significant change in live cell number was seen with either cell type. However, when gels were released (at day-4) and allowed to contract (for 3-days, to day-7) a significant drop in live cell number (~30-40%, p=0.016) was detected in gels seeded with normal scar fibroblasts. In contrast this was not seen in the gels seeded with keloid scar fibroblasts (p=0.512), where the live cell number remained relatively constant.

Fibrin matrices were set up in a similar manner to that of the stressed-relaxed collagen gel model. Results from normal scar and keloid scar fibroblasts seen in Figure 3.9A and B, show no significant drop in live cell number in either of the gel models (anchored or contractile) over the entire 7-day time course. However, there was a significant increase in live cell number in the contractile fibrin matrices seeded with both normal scar fibroblasts and keloid scar fibroblasts (p= 0.025 and p= 0.031, correspondingly) from day-0 to day-7 (Figure 3.9A and B). The effect of fibrin and collagen matrix culture on normal scar cells appears to elicit opposing effects, with a contractile collagen matrix inducing cell death and a contractile fibrin matrix encouraging cell proliferation. Whilst induction of proliferation by fibrin matrices was obvious for keloid scar-derived fibroblasts and equivalent to that seen with normal scar cells, no induction of cell death of keloid scar cells was seen in contractile collagen matrices even when contraction was continued for up to 10-days (not shown).

These results confirm the morphological findings, that unlike normal scar fibroblasts, keloid scar fibroblasts do not respond to the apoptosis-inducing effects of contractile collagen matrix culture. Although this defect is clearly not simply due to an inability of keloid scar fibroblasts to contract collagen gels (Figure 3.3 and 3.4) it is possible that contraction differs in a more qualitative manner, which might affect the induction of apoptosis. The percentage of apoptosis in collagen gels only reaches ~30% which indicates that only a certain population of cells are able to respond, or are susceptible to the apoptosis cues.

A. Normal Scar



B. Keloid Scar

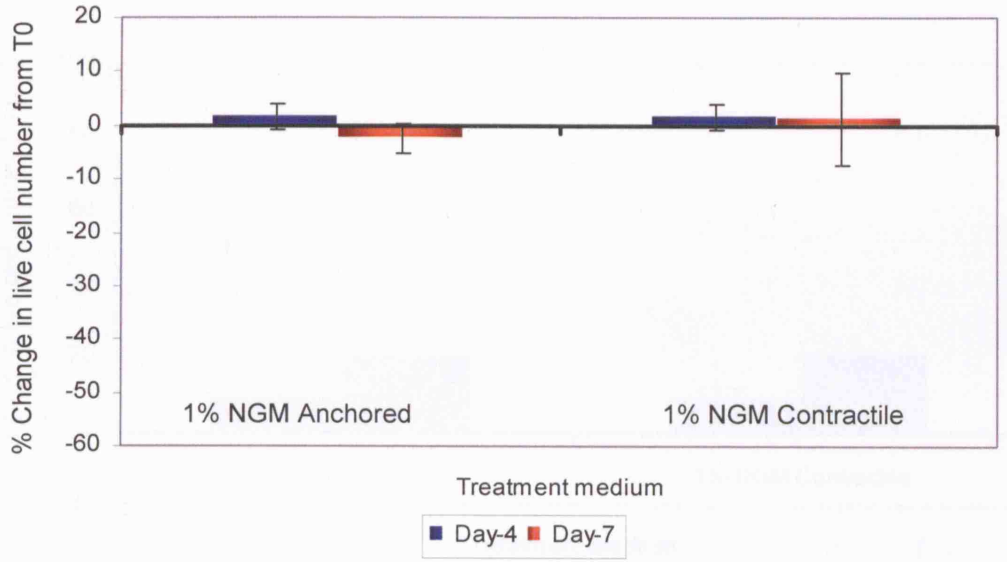
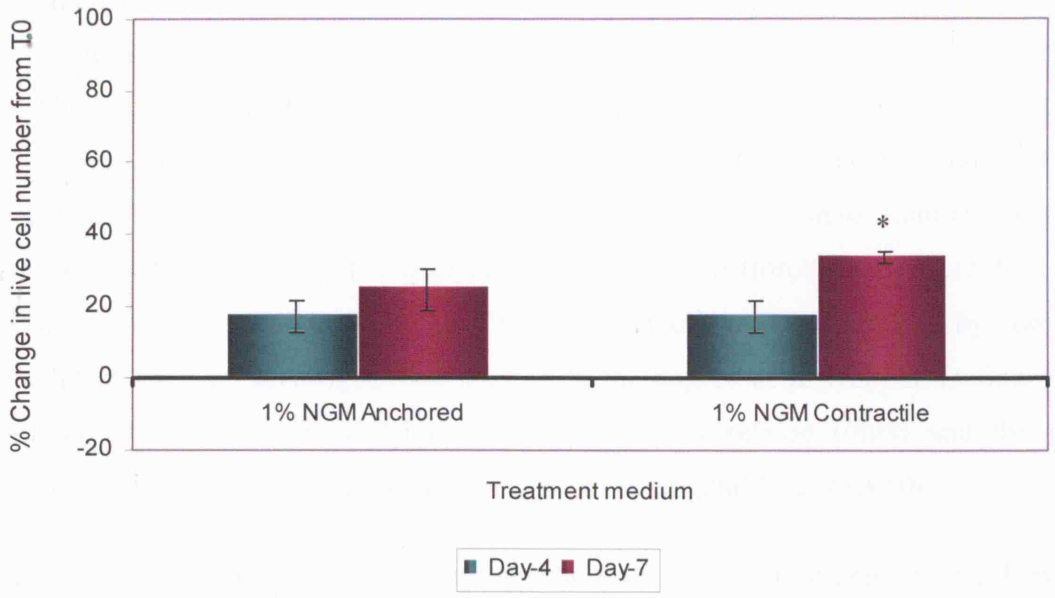


Figure 3.8. Fibroblasts derived from normal scar or keloid scar were seeded into 3-D collagen gels. At day-0 (24hrs after seeding gels prior to adding 1% NGM), -4 and -7 live cell number was assessed in anchored (stressed) and contractile (relaxed) matrices. Results plotted represent the percentage change in live cell number from day-0 (T0). Results are expressed as a mean of the experiment performed in triplicate with n=4 cell strains for each scar type. Error bars represent SD. T-test analysis compared the percentage change in live cell numbers from day-7 anchored matrices to day-7 contractile matrices. *P=<0.05.

A. Normal Scar



B. Keloid Scar

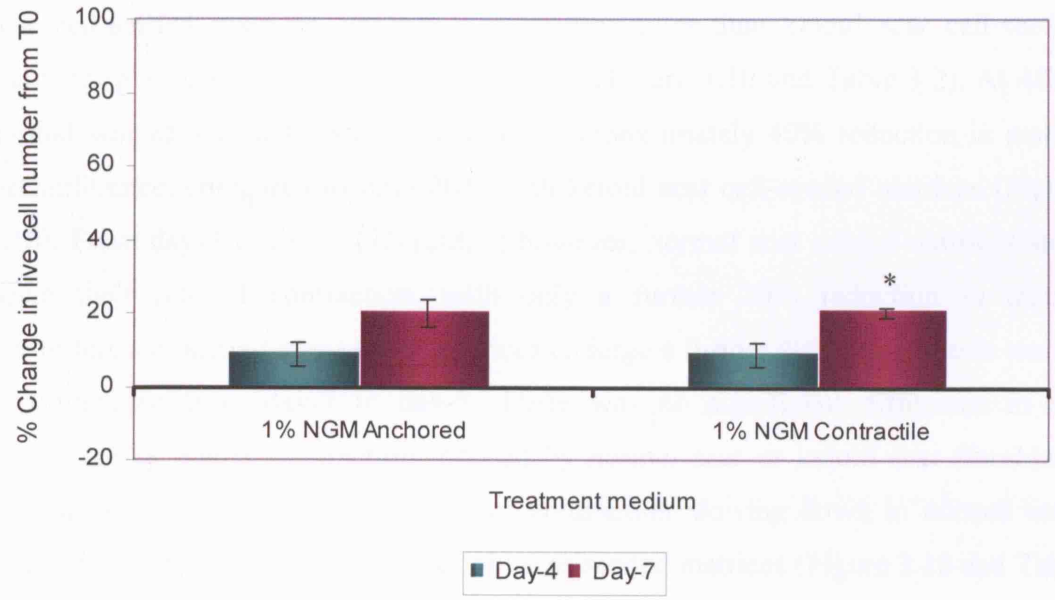


Figure 3.9 Fibroblasts derived from normal scar or keloid scar were seeded into 3-D fibrin gels. At day-0 (24hrs after seeding gels prior to adding 1% NGM), -4 and -7 live cell number was assessed in anchored (stressed) and contractile (relaxed) matrices. Results represent the percentage change in live cell number from day-0. Results are expressed as a mean of the experiment performed in triplicate with n=4 cell strains for each scar type. Error bars represent SD. T-test analysis compared the percentage change in live cell numbers from day-7 anchored matrices to day-7 contractile matrices. *P=<0.05.

3.2.4 The Ability of Normal Scar and Keloid Scar Fibroblasts to Contract 3-D Collagen Matrices

As discussed in section 3.1.3 fibroblasts within stress-relaxed matrices act to contract the matrix through both tractional forces (cell organisation) and cell-contraction (cell shortening). It is possible that keloid scar fibroblasts may fail to undergo one of these mechanisms. Keloid scar fibroblasts may cause matrix contraction to occur at a slower rate, but eventually reaching that of normal scar-derived fibroblasts (Figure 3.4), or the matrix contraction that does occur is not as effective as that induced by normal scar fibroblasts. To investigate this possibility the degree of matrix contraction was assessed over 5-days from the time-point of matrix release (0hrs) and the gel circumference was expressed as a percentage of the original (Figure 3.10).

Figure 3.10 shows that although a similar final level of matrix contraction is achieved for normal scar cell and keloid scar cell-seeded matrices, the pattern of contraction differs over time. During the first 2-days (4, 24, 48hrs) of matrix contraction, normal scar cell-seeded matrices contract significantly more than keloid scar cell-seeded matrices ($p=0.024$, 0.023, 0.050, respectively) (Figure 3.10 and Table 3.2). At 48hrs normal scar cell-seeded matrices underwent approximately 40% reduction in matrix circumference, compared to only 20% with keloid scar cell-seeded matrices (Figure 3.10). From day-3 to day-5 (72-120hrs) however, normal scar seeded-matrices slow down their rate of contraction, with only a further 20% reduction in matrix circumference. Keloid scar seeded matrices undergo a further 40% reduction in matrix circumference from day-3 to day-5. There was no significant difference in the percentage of matrix contraction induced by normal scar or keloid scar fibroblasts from day-3 to day-5, due to the rate of contraction slowing down in normal scar-seeded matrices, and increasing in keloid scar-seeded matrices (Figure 3.10 and Table 3.2). This agrees with the results in Figure 3.4, where no significant difference in contraction between normal scar and keloid scar cell-seeded matrices was found after 3-days of contraction. Photographs in Figure 3.11 illustrate the trend of increased matrix contraction with normal scar cell-seeded matrices at approximately 24-48hrs compared to keloid scar cell-seeded matrices, with the difference between the two becoming less evident with time.

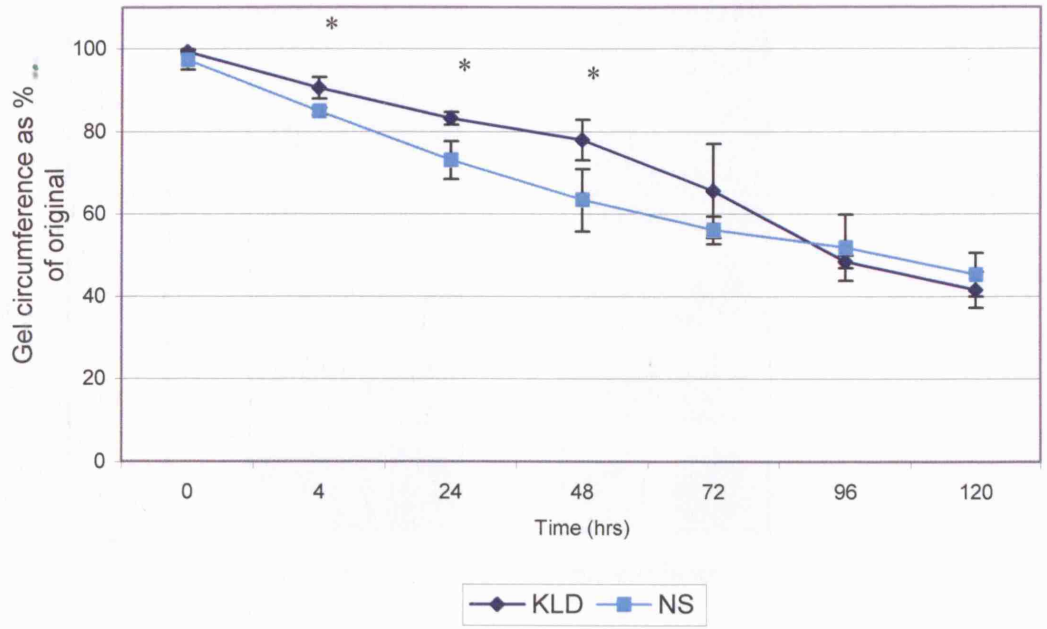


Figure 3.10. Measurement of the degree of contraction induced by normal scar (NS) or keloid scar (KLD) fibroblasts. Cells were embedded into collagen matrices and maintained in 1% NGM. The matrices were retained anchored for a period of 4-days prior to being released. At the point of gel release (day-4) the circumference of the matrix was measured (0hrs) as well as the circumference of the well. The degree of matrix contraction was measured periodically over 5-days and was calculated as the percentage change in matrix circumference from the original circumference of the well. Results represent a mean of the experiment performed with n=4 cell strains for each scar type, each carried out in duplicate. Error bars represent SD. T-test analysis compared the degree on contraction induced by normal scar vs that by keloid scar fibroblasts. *P=<0.05.

Table 3.2 Statistical analysis of normal scar vs keloid scar fibroblast induced collagen matrix contraction. Collagen matrices were set up as normal, retaining the matrices anchored for a period of 4-days before releasing them to allow contraction. The degree of matrix contraction was measured from the time-point of gel release (0hrs) periodically over 5-days (0-120hrs) and was calculated as the percentage change in matrix circumference from the original circumference of the well. Data represents P-values after T-test analysis of raw data. *P=<0.05.

Mean % Contraction (± SD)							
	0hrs	4hrs	24hrs	48hrs	72hrs	96hrs	120hrs
NS	97.4 ± 2.3	84.8 ± 0.9	73.1 ± 4.6	63.4 ± 7.6	56.1 ± 3.4	51.9 ± 8.0	45.3 ± 5.3
KLD	99.3 ± 0.6	90.5 ± 2.6	83.1 ± 1.5	78.0 ± 4.8	65.6 ± 11.5	48.4 ± 1.5	41.7 ± 4.3
NS vs KLD	P=0.231	**P=0.024***	**P=0.023***	**P=0.050***	P=0.240	P=0.507	P=0.404

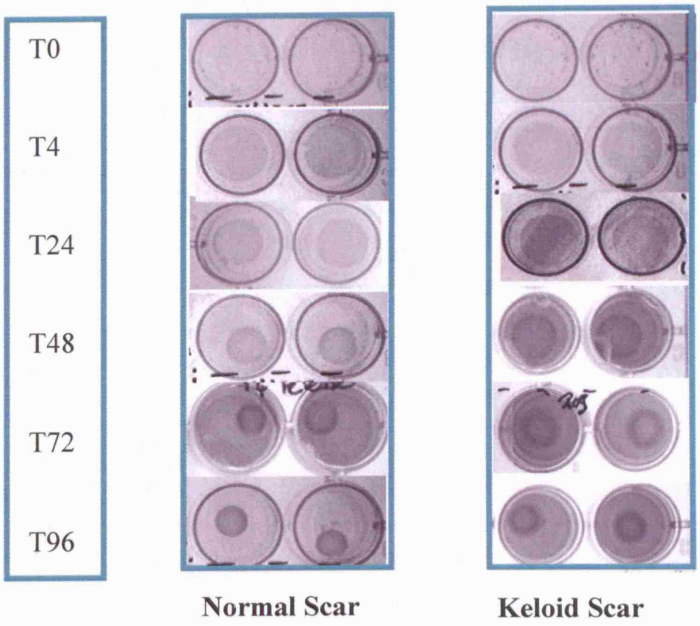


Figure 3.11 Photographic representations of stress-relaxed matrices after retaining the matrices anchored for 4-days prior to matrix release. The matrices were seeded with keloid scar- or normal scar-derived fibroblasts, cultured in 1% NGM. Photographs were taken at 0, 4, 24, 48, 72 and 96hrs (T0-96). This Figure represents n=1 normal scar and keloid scar cell strains. Results are typical of n=4 normal scar and keloid scar cell strains.

This result suggests that keloid scar fibroblasts do contract a collagen matrix eventually to the same extent but perhaps not in the same manner as that of normal scar fibroblasts. This may be through decreased cell-matrix interactions or the type of contraction elicited by the cells or even a change in rigidity of the collagen matrix brought about by the keloid cells. What is clear is that the contraction that is produced by keloid scar fibroblasts is not as effective at inducing apoptosis as that produced by normal scar fibroblasts

3.2.5 The Effect of Chemical Apoptosis Induction on Keloid Scar Fibroblasts

The results displayed in this chapter so far clearly demonstrate a defect in the ability of keloid scar fibroblasts to undergo apoptosis under the conditions of a laboratory model that mimics the wound healing situation. It is therefore important to establish whether this phenomenon is caused by the inability to produce or respond to specific wound healing (collagen-contraction) cues of apoptosis or due to a general/universal fault in the apoptotic mechanism within these cells.

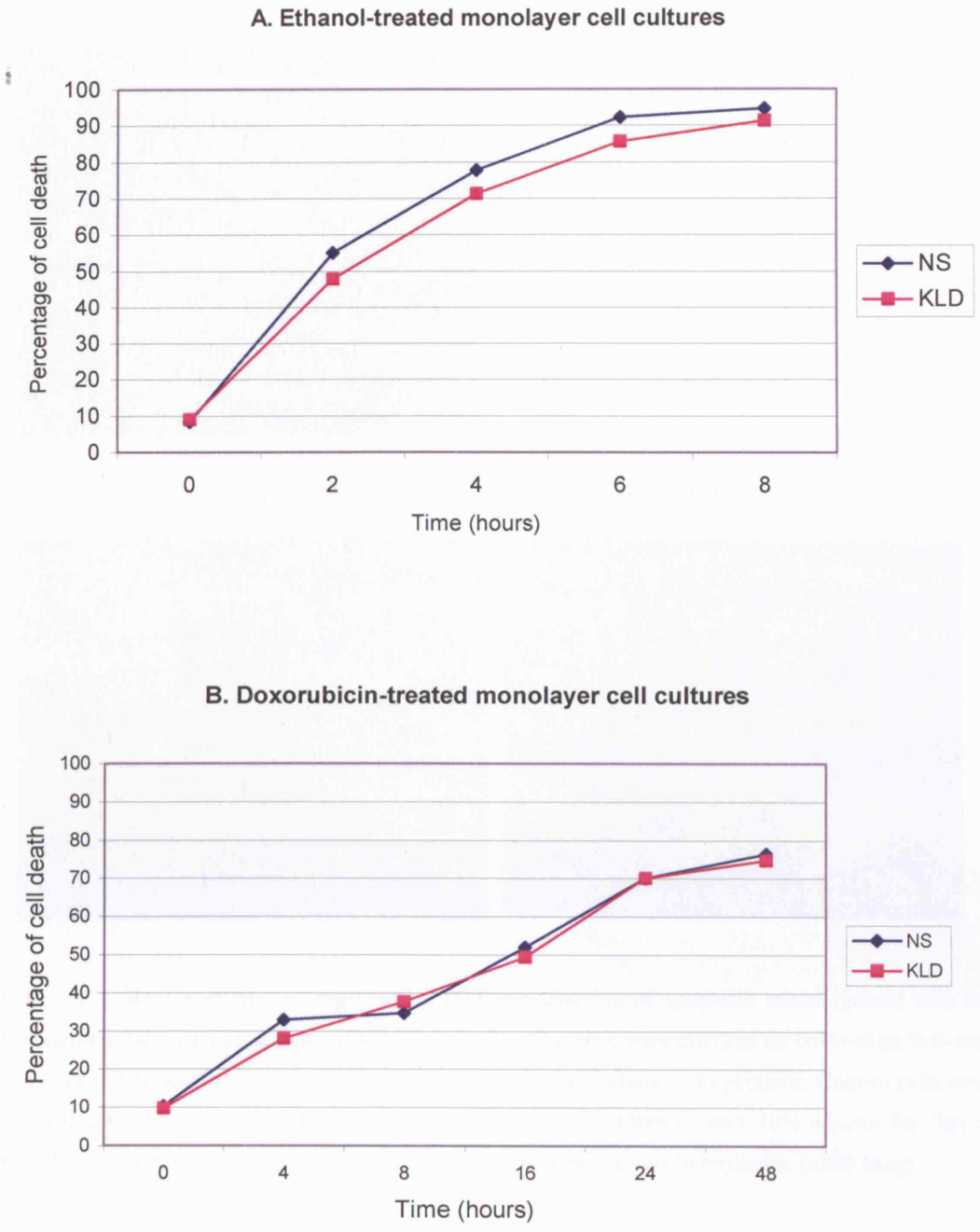


Figure 3.12 Cytotoxic time-course analysis assessing the percentage of cell death over time between normal scar (NS) and keloid scar (KLD) derived fibroblast monolayer cultures. Cells were seeded onto monolayer 6-well tissue culture grade plates in NGM and maintained for 24hrs before treating with 10% ethanol or doxorubicin (1mg/ml) in SFM for 8 and 48hrs correspondingly. Viable cell counts were performed at specific time-points. This result represents the mean of the experiment performed in triplicate with n=3 cell strains for each scar type.

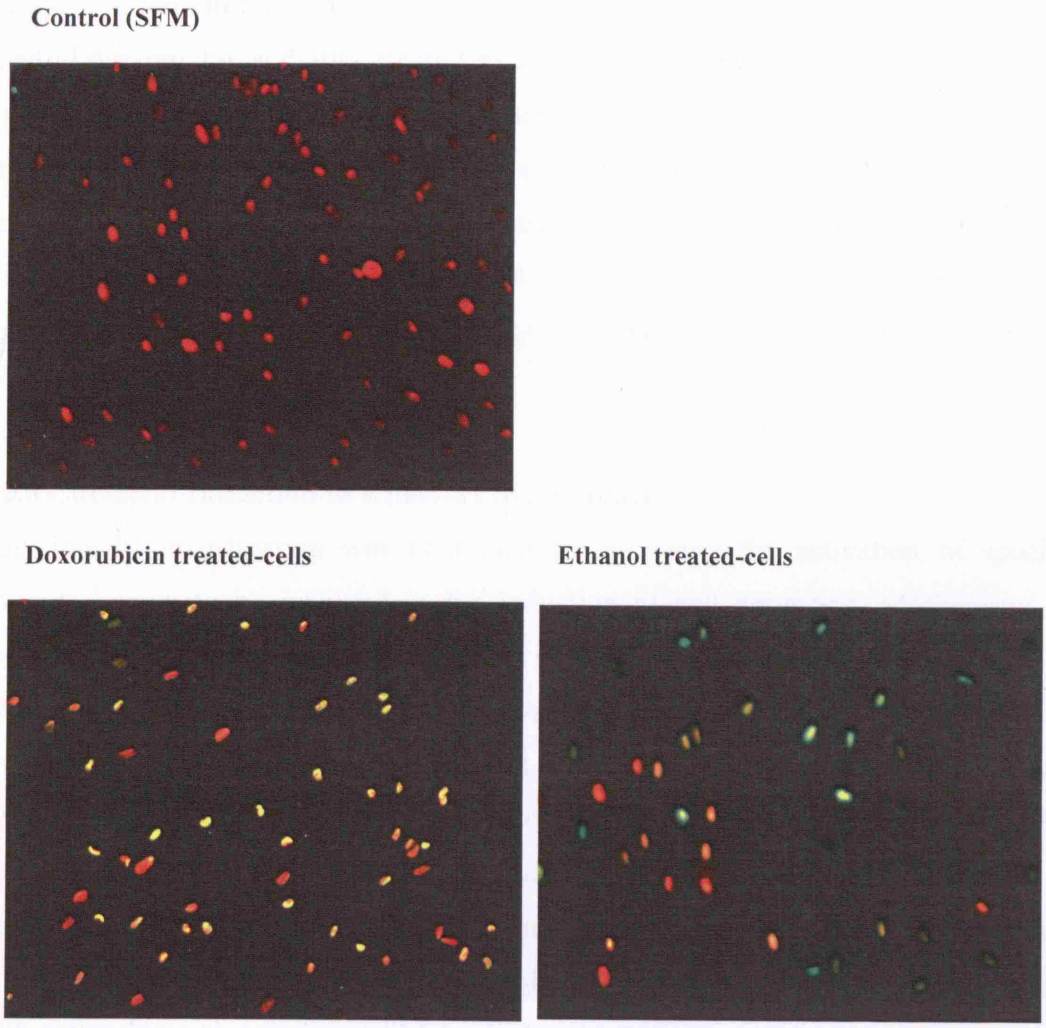


Figure 3.12C Representative micrographs of TDT end-labelling of apoptotic nuclei (green) with red (propidium iodide) counterstaining of keloid scar cells. The cells were cultured on cover-slips in 6-well plates, once 70% confluent the cells were treated with known inducers of apoptosis. Control cells were untreated, test cells were treated with 1mg/ml doxorubicin for 48hrs or with 10% ethanol for 7hrs in SFM. The experiment was carried out with n=5 keloid scar cell strains in triplicate. (x200 Mag).

To investigate this anomaly, monolayer cultured fibroblasts derived from keloid scar were subjected to chemical induction of apoptosis. Cells were treated with the cytotoxic drug doxorubicin for up to 48hrs or 10% ethanol treatment for up to 7hrs (experiments previously optimised in the laboratory at RAFT); both methods of cell death induction are known to specifically induce apoptosis via caspase activation (Vaculova *et al.*, 2004; Valles *et al.*, 2004; Casares *et al.*, 2005; Jurisicova *et al.*, 2006).

Titrated concentrations of doxorubicin and ethanol determined that a concentration of 1mg/ml doxorubicin and 10% ethanol in SFM caused maximum apoptosis after 48hrs and 7 hrs, correspondingly (personal communication - Dr Claire Linge). In addition, time course analysis showed that there was no apparent difference between either scar cell type in sensitivity to apoptosis induction over time with ethanol (Figure 3.12A) and doxorubicin treatment (Figure 3.12B). Figure 3.12C shows TdT end-labelling with the ApoBrdU kit, which demonstrates that the cell death observed was due to apoptosis in keloid scar cells.

3.2.6 Chemical Induction of Effectors of Apoptosis

This line of investigation was continued by assessing the activation of specific proteins known to be involved in the induction of cell apoptosis. Mechanisms of apoptosis are discussed in greater detail in section 1.6. Western blotting was used to assess a number of important apoptosis-related proteins; the check-point protein involved in assessing and signalling cells to undergo apoptosis (p53) (Rich *et al.*, 2000), the main death effector protease of the cell (caspase-3) (Takahashi, 1999; Hengartner, 2000) pivotal to apoptosis induction, and the main cleavage protein of caspase-3 (PARP) which is situated within the cells nucleus. In order to induce the activation of these proteins, cells were again cultured to 70% confluence and treated with doxorubicin (1mg/ml) in SFM for 0, 8 and 24hrs. These time points were chosen after carrying out optimisation assays with Jurkat cells, which showed sufficient caspase-3 activation after 8 and 24hrs of doxorubicin treatment (see Appendix XI). The expression of a constitutively expressed house-keeping gene (GAPDH) was assessed by western blot analysis, in addition to the proteins of interest, as a loading control. Specific protein expression was calculated as a ratio of densitometric quantification of the protein band of interest to that of the GAPDH loading control.

p53

Figure 3.13A, B and Table 3.3A, clearly shows a significant increase in the level of p53 in normal scar- and keloid scar-derived fibroblasts ($p=0.048$ and $p=0.012$, respectively) after 8hrs treatment with the cytotoxic drug doxorubicin. This represents a stabilisation of the protein, which is typical after cytotoxic insult (see section 1.6.1), as it is normally rapidly turned over.

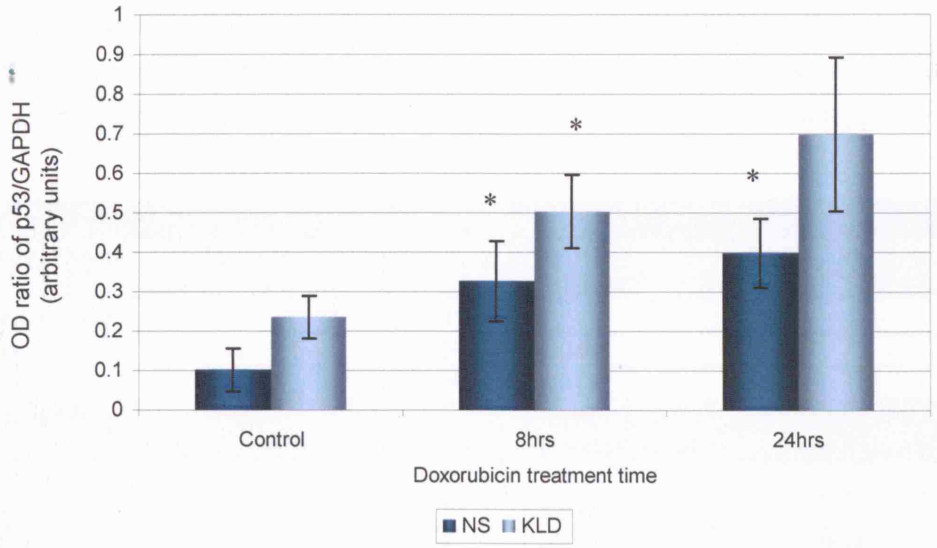
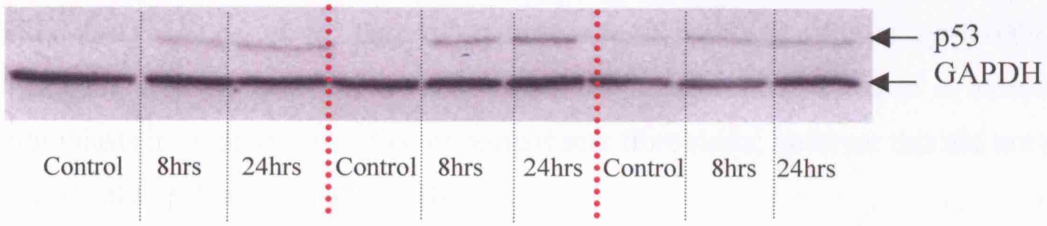


Figure 3.13A Normal scar (NS) and keloid scar (KLD) fibroblasts were induced to undergo p53 stabilisation by treating confluent monolayers with 1mg/ml doxorubicin in SFM (known to induce apoptosis) for 0, 8 and 24hrs. Results from western blotting for p53 where analysed by densitometry. p53 levels are expressed as a ratio to that of the house-keeping gene (GAPDH). Each bar represents the mean of n=3 cell strains (each performed in triplicate) for each scar type. Error bars represent SD. Paired T-test analysis compared p53 stabilisation in cells after 8hrs and 24hrs of doxorubicin treatment compared to that detected in control cells. *P=<0.05.

Normal scar cells



Keloid scar cells

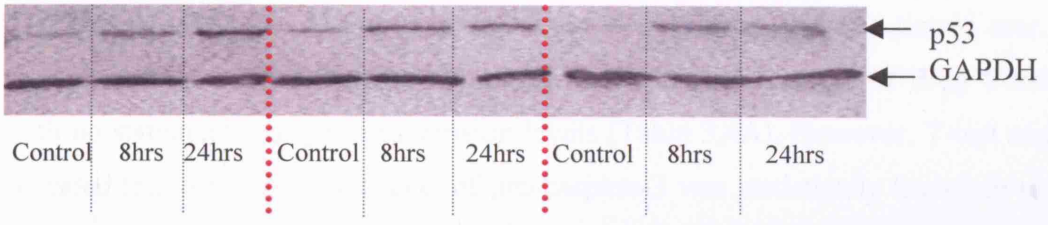


Figure 3.13B Western blot analysis of p53 stabilisation (53KDa) with 3x normal scar and 3x keloid scar cell strains treated with 1mg/ml doxorubicin for 0, 8 and 24hrs. The pink line separates the different cell strains.

Table 3.3 Statistical data from densitometric analysis of western blots for p53 stabilisation after doxorubicin drug treatment. Table A represents p-values after paired T-test analysis of raw data and Table B represents p-values after T-test analysis of raw data. Results presented in Tables A and B represents data from n=3 cell strains (each performed in triplicate) for each scar type. *P=<0.05.

A. Paired T-Test				
Normal Scar	**Mean**	**± SD**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.102	± 0.05		
8hrs	0.327	± 0.10	**0.048***	
24hrs	0.398	± 0.09	**0.049***	0.318
Keloid Scar	**Mean**	**± SD**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	9.85	± 6.9		
8hrs	20.65	± 5.7	**0.012***	
24hrs	24.10	± 2.3	0.080	0.342

B. T-Test Keloid vs Normal Scar Fibroblasts			
	0hrs	**8hrs**	**24hrs**
p53	**0.030***	0.090	0.068

After 24hrs of drug treatment, both scar types exhibited a 4-fold increase in p53 activation in comparison to that of non-treated, control fibroblasts. Densitometry and statistical analysis (Figure 3.13B and Table 3.3B) revealed that the basal expression level of p53 in control cells appears to be significantly higher in keloid scar-derived fibroblasts compared to that of normal scar-derived fibroblasts (p=0.030). On treatment with doxorubicin the p53 expression levels remain elevated in keloid scar fibroblasts in comparison to that of normal scar fibroblasts; however this did not reach statistical significance (Table 3.3B).

Caspase-3

The expression levels of pro-caspase-3 seen with normal scar and keloid scar cells (Figure 3.14) remained relatively constant over the time-course of drug treatment, with no statistical change in expression levels (Table 3.4A). However, T-test analysis revealed that the expression level of pro-caspase-3 was statistically higher in normal scar-derived cell lysates compared to that of keloid scar cell lysates at both the basal level (p=0.01) and after 8 and 24hrs of drug treatment (p=0.012 and 0.002, respectively) (Table 3.4A).

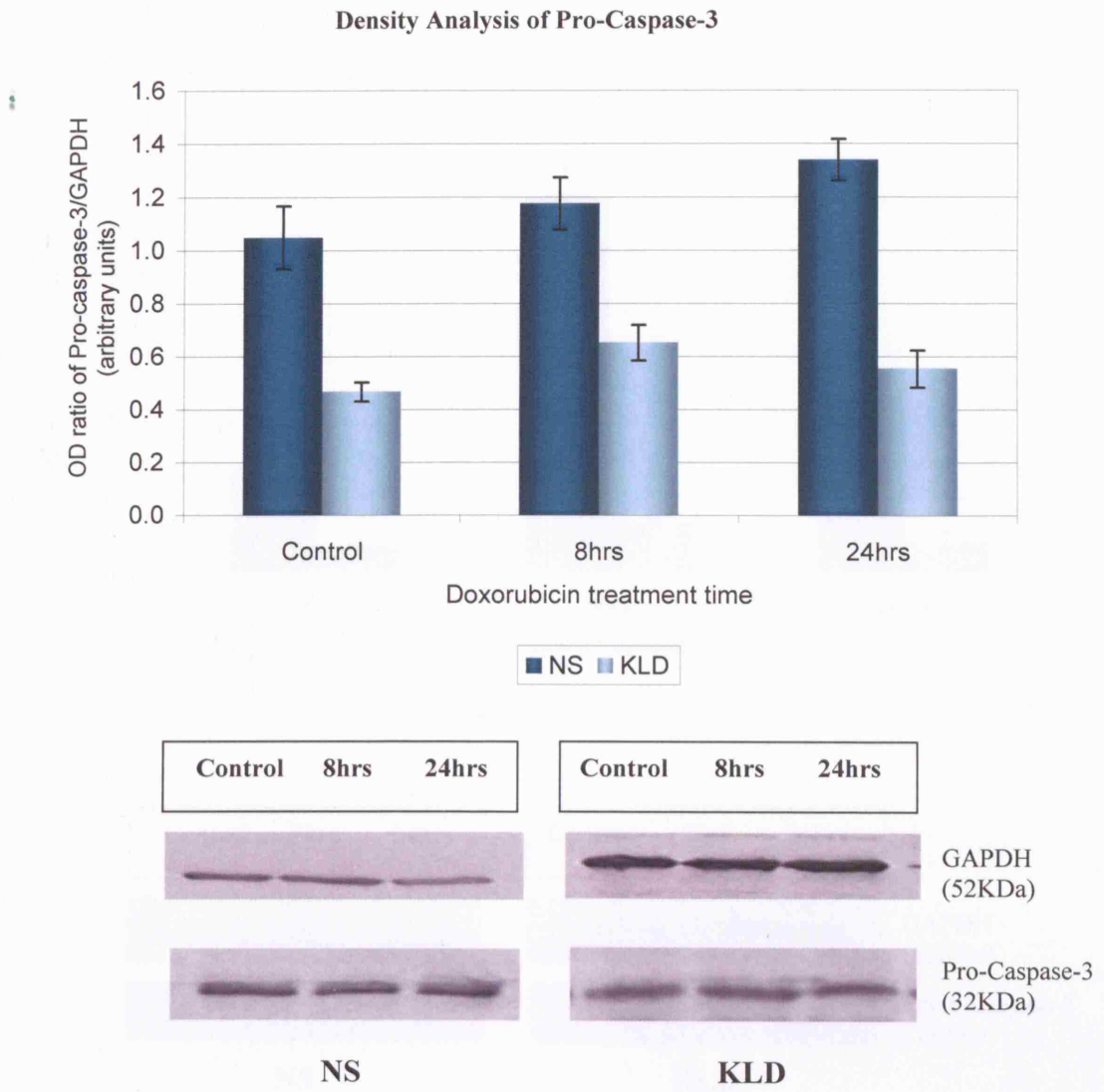


Figure 3.14 Densitometry analysis of pro-caspase-3 expression after western blot analysis. Normal scar (NS) and keloid scar (KLD) fibroblasts were treated with 1mg/ml doxorubicin for 0, 8 and 24hrs in SFM. Pro-caspase-3 levels are expressed as a ratio to that of the house-keeping gene (GAPDH). Each bar represents the mean of n=3 cell strains (each performed in triplicate) for each scar type. Error bars represent SEM. Paired T-test analysis compared levels of pro-caspase-3 in cells after 8hrs and 24hrs of doxorubicin treatment compared to that detected in control cells.

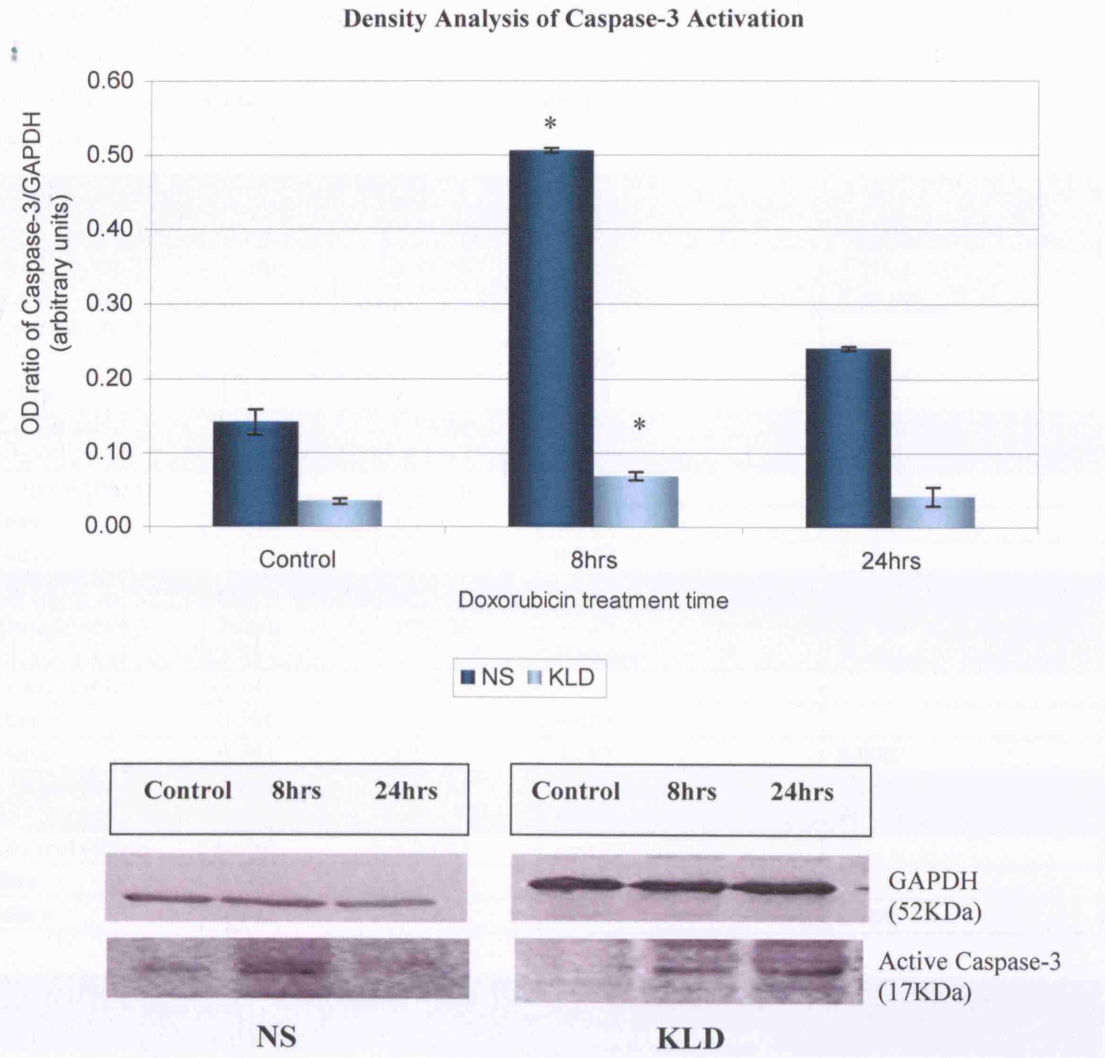


Figure 3.15 Densitometry analysis of caspase-3 activation after western blot analysis. Normal scar (NS) and keloid scar (KLD) fibroblasts were treated with 1mg/ml doxorubicin for 0, 8 and 24hrs in SFM. Active caspase-3 levels are expressed as a ratio to that of the house-keeping gene (GAPDH). Each bar represents the mean of n=3 cell strains (each performed in triplicate) for each scar type. Error bars represent SEM. Paired T-test analysis compared caspase-3 activation in cells after 8hrs and 24hrs of doxorubicin treatment compared to that detected in control cells. *P=<0.05.

Table 3.4 Statistical data from densitometric analysis of western blots for Pro-caspase-3 and caspase-3 activation after doxorubicin drug treatment. Table A represents p-values after paired T-test analysis of raw data and Table B represents p-values after T-test analysis of raw data. Results presented in Tables A and B represents data from n=3 cell strains (each performed in triplicate) for each scar type. *P=<0.05 and **P=<0.001.

A. Paired T-Test				
Pro-Caspase-3				
Normal scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	1.048	± 0.12		
8hrs	1.176	± 0.09	0.455	
24hrs	1.339	± 0.07	0.111	0.264
Keloid scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.467	± 0.036		
8hrs	0.652	± 0.67	0.074	
24hrs	0.554	± 0.07	0.347	0.381
Active Caspase-3				
Normal scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.142	± 0.01		
8hrs	0.506	± 0.03	**0.029***	
24hrs	0.241	± 0.03	0.195	**0.008***
Keloid scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.034	± 0.003		
8hrs	0.069	± 0.01	**0.045***	
24hrs	0.042	± 0.01	0.725	0.169

B. T-Test			
Keloid vs Normal Scar Fibroblasts			
	0hrs	**8hrs**	**24hrs**
Pro-Caspase-3	0.010	0.012	0.002
ActiveCasp-3	0.072	**<0.001****	**0.005***

Activation of caspase-3 was measured by the appearance of the cleaved 17kDa active fragment of caspase-3. After 8hrs of doxorubicin treatment a significant increase in activated caspase-3 was detected in both normal scar- and keloid scar-derived fibroblasts (p=0.029 and p=0.045, respectively) (Figure 3.15 and Table 3.4A). In addition, after 8hrs of drug treatment normal scar fibroblasts underwent a 3-fold increase in active caspase-3 levels, this then decreased almost to untreated levels after 24hrs of treatment, which was not significantly different (p= 0.195) to T0 control cells (Figure 3.15 and Table 3.4A). This significant drop in activation from 8 to 24hrs (p=0.008) is possibly due to the very short half-life of active caspase-3 or due to the fact that ~70% of the cells are dead as shown in Figure 3.12 and the expression of active caspase-3 detected is from the remaining live cells.

Keloid scar-derived fibroblasts also followed the same activation pattern, but not to the same levels, with active caspase-3 levels increasing by 2-fold after 8hrs of drug treatment. After 24hrs, the level of active caspase-3 failed to reach significance (p= 0.725) in comparison to T0 control fibroblasts (Figure 3.15 and Table 3.4A). Although the basal expression of active caspase-3 in the control cells (untreated) appears higher in normal scar fibroblasts in comparison to keloid scar fibroblasts, with a 4-fold difference, this is not statistically significant. However, on drug treatment normal scar cells do exhibit a significantly increased level of active caspase-3 in comparison to that of keloid scar fibroblasts at 8 and 24hrs (7-fold difference p= <0.001 and 5-fold difference p=0.005, correspondingly) (Table 3.4B). This apparent increased activation of caspase-3 may simply be due to the significantly higher expression levels seen with normal scar fibroblasts. Nevertheless, cell death appeared to be equally induced by this drug treatment in both normal scar and keloid scar cells.

PARP

On assessing the expression of latent PARP in normal scar and keloid scar fibroblasts after drug treatment, no significant change in the protein levels was detected, although normal scar and keloid scar fibroblasts both displayed a trend of increasing latent PARP expression after 8hrs of doxorubicin treatment (Table 3.5A). In addition, there was no significant difference in latent PARP expression between keloid and normal scar-derived cells in untreated or treated samples (Figure 3.16 and Table 3.5B).

As with the activation of caspase-3, PARP activation is again significantly higher in normal scar fibroblasts compared to keloid scar fibroblasts in untreated control cells (14-fold difference, p=<0.001) and after 8hrs of drug treatment (though markedly less pronounced being only a 2-fold difference, p=0.041, respectively) (Table 3.5B). Figure 3.17 and Table 3.5A shows that PARP activation in normal scar fibroblasts steadily increases by approximately 2-fold over the treatment time, reaching significance after 24hrs of drug treatment (p=0.035). Keloid scar-derived fibroblasts however, show a significant increase in PARP activation by 8hrs (p=0.028) with approximately a 20-fold increase in expression levels. Active PARP levels continue to rise after 24hrs of drug treatment however; levels never reach those expressed by normal scar cells (Figure 3.17).

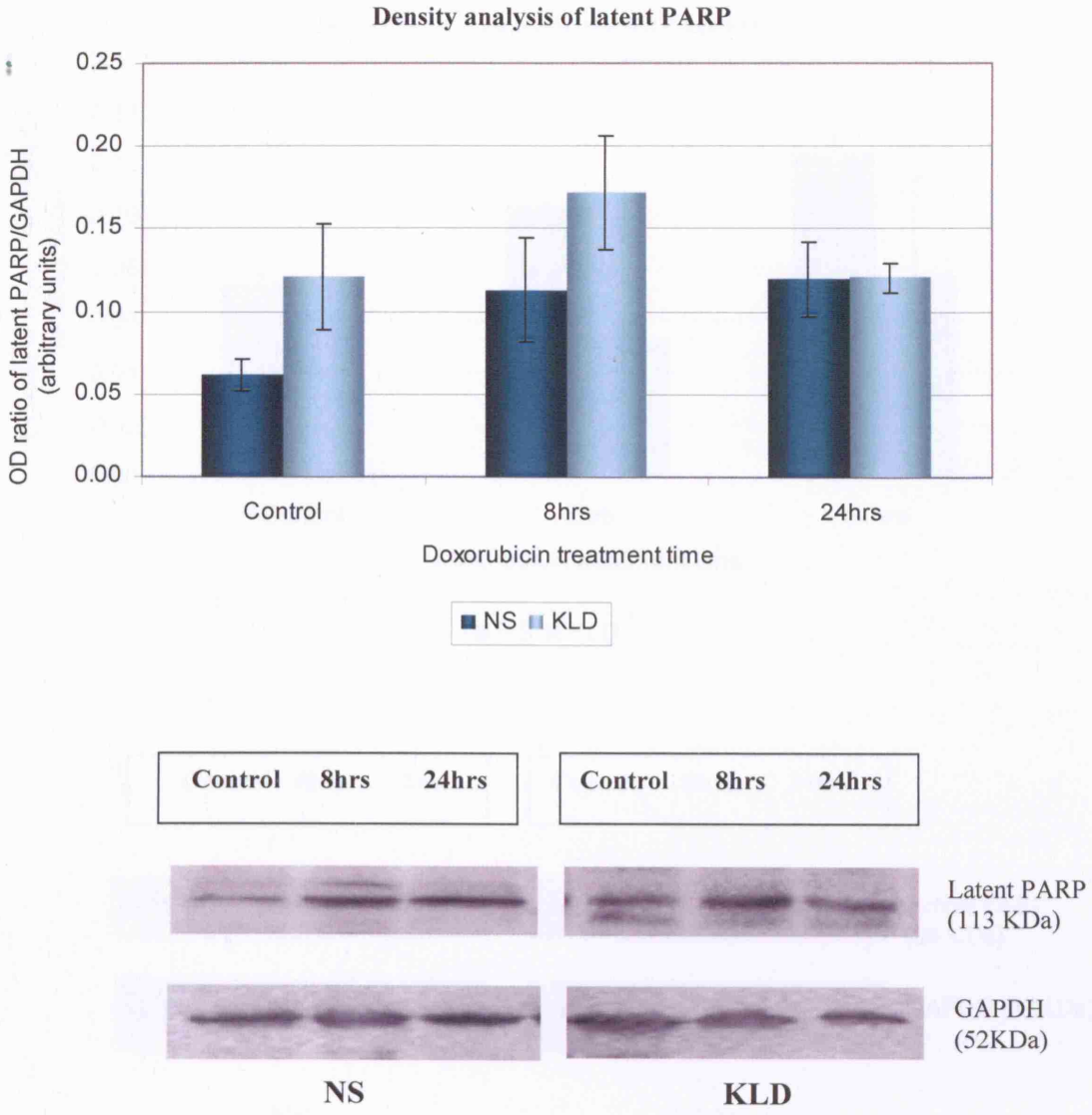


Figure 3.16 Densitometry analysis of latent PARP levels after western blot analysis. Normal scar (NS) and keloid scar (KLD) fibroblasts were treated with 1mg/ml doxorubicin for 0, 8 and 24hrs in SFM. Latent PARP levels are expressed as a ratio to that of the house-keeping gene (GAPDH). Each bar represents the mean of n=3 cell strains (each performed in triplicate) for each scar type. Error bars represent SEM.

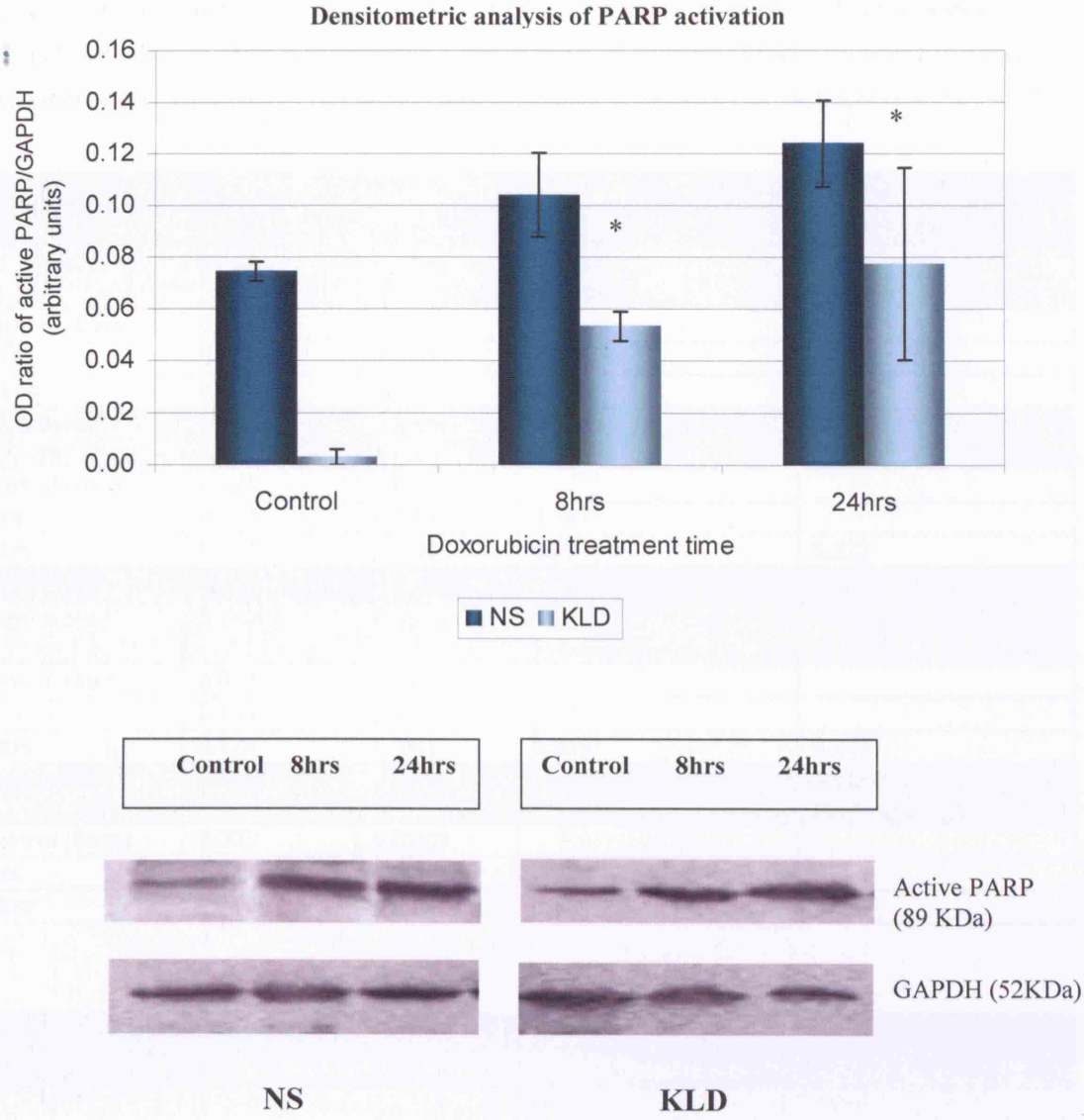


Figure 3.17 Densitometry analysis of PARP activation after western blot analysis. Normal scar (NS) and keloid scar (KLD) fibroblasts were treated with 1mg/ml doxorubicin for 0, 8 and 24hrs in SFM. Active PARP levels are expressed as a ratio to that of the house-keeping gene (GAPDH). Each bar represents the mean of n=3 cell strains (each performed in triplicate) for each scar type. Error bars represent SEM. Paired T-test analysis compared PARP activation in cells after 8hrs and 24hrs of doxorubicin treatment compared to that detected in control cells. *P=<0.05.

Table 3.5 Statistical data from densitometry analysis of western blots for latent PARP and PARP activation after doxorubicin treatment. Table A represents p-values after paired T-test analysis of raw data and Table B represents p-values after T-test analysis of raw data. Results presented in Tables A and B represents data from n=3 cell strains (each performed in triplicate) for each scar type. *P=<0.05.

A. Paired T-Test				
Latent PARP				
Normal scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.041	± 0.01		
8hrs	0.113	± 0.03	0.206	
24hrs	0.120	± 0.02	0.096	0.876
Keloid scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.121	± 0.03		
8hrs	0.172	± 0.03	0.347	
24hrs	0.121	± 0.01	0.993	0.222
Active PARP				
Normal scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.074	± 0.003		
8hrs	0.104	± 0.01	0.272	
24hrs	0.124	± 0.01	**0.035***	**0.441**
Keloid scar	**Mean**	**± SEM**	**Vs 0hr P-value**	**Vs 8hr P-value**
Control (0hrs)	0.003	± 0.003		
8hrs	0.053	± 0.01	**0.028***	
24hrs	0.077	± 0.04	0.164	0.634

B. T-Test			
Keloid vs Normal Scar Fibroblasts			
	0hrs	**8hrs**	**24hrs**
Latent PARP	0.176	0.273	0.969
Active PARP	<0.001**	0.041*	0.216

These results demonstrate that keloid scar fibroblasts are able to respond to the apoptotic induction signals induced by cytotoxic insult.

3.2.7 The Induction of Caspase-3 after Collagen Matrix Contraction

Results from the chemical induction of apoptosis, show that there is not a universal fault in the ability of keloid scar fibroblasts to undergo apoptosis. Caspase-3 activation during collagen matrix contraction was investigated in normal scar and keloid scar cell-seeded matrices.

This death-associated protein was chosen for further investigation due to its potential role in wound healing-related apoptosis as a result of its direct activation by potential products of matrix remodelling (section 4.1.2).

Western blot analysis was used to assess caspase-3 activation in normal scar and keloid scar cell-protein lysates after collagen matrix culture (Figure 3.18A and B). Densitometry analysis of western blots for n=3 of each cell strain revealed that caspase-3 is significantly ($p=<0.001$) activated specifically on collagen matrix contraction (Figure 3.18B) by normal scar-derived fibroblasts, but not by keloid scar-derived fibroblasts. This coincides with a trend of decreasing pro-caspase-3 expression in normal scar fibroblasts (as also seen in keloid scar-derived fibroblasts), which failed to reach significance (Figure 3.18A and B).

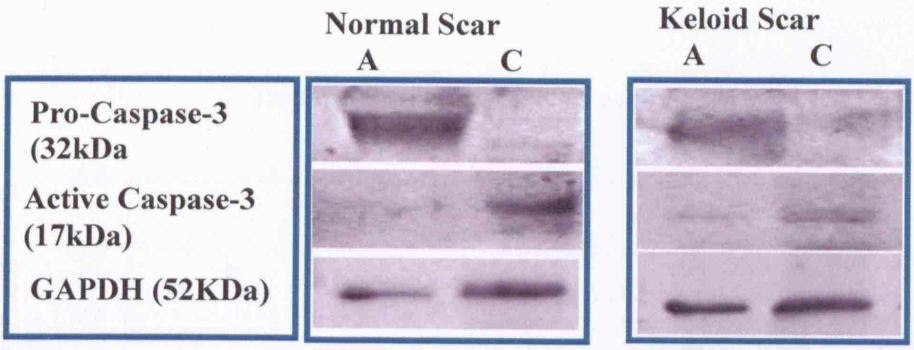
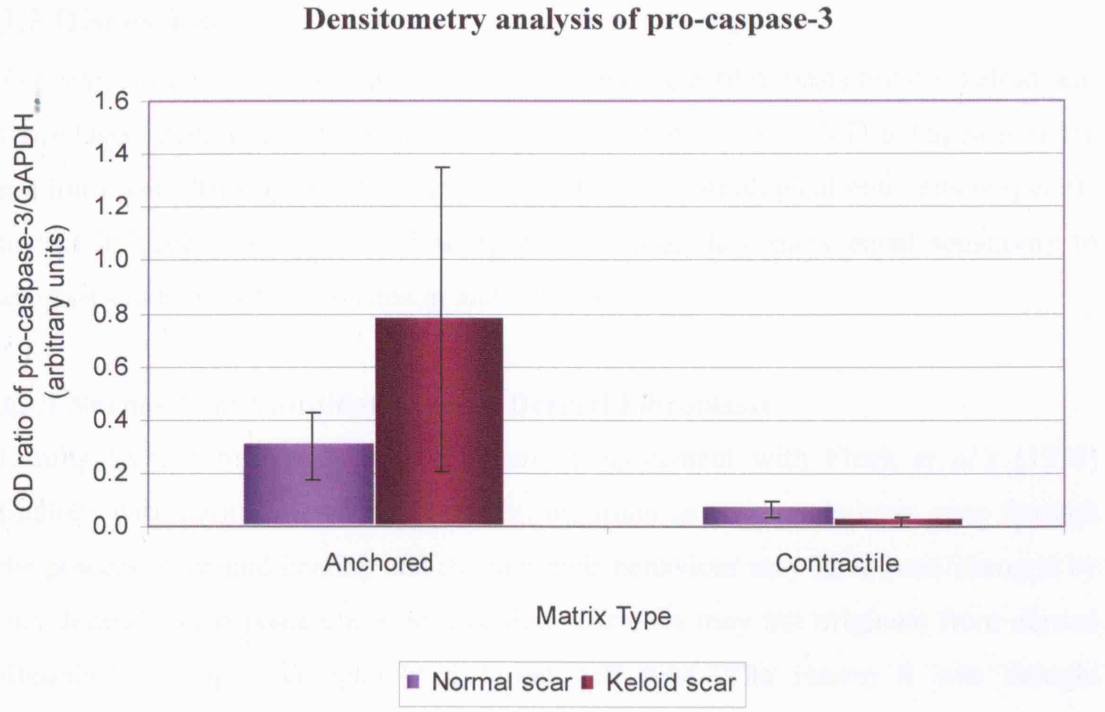


Figure 3.18A Typical western blot of caspase-3 activation and GAPDH (house-keeping gene) by normal scar and keloid scar cells after 7-days of collagen gel culture in anchored (A) or contractile matrices (C). These result are representative of experiments carried out in triplicate with n=3 scar strains per scar type.



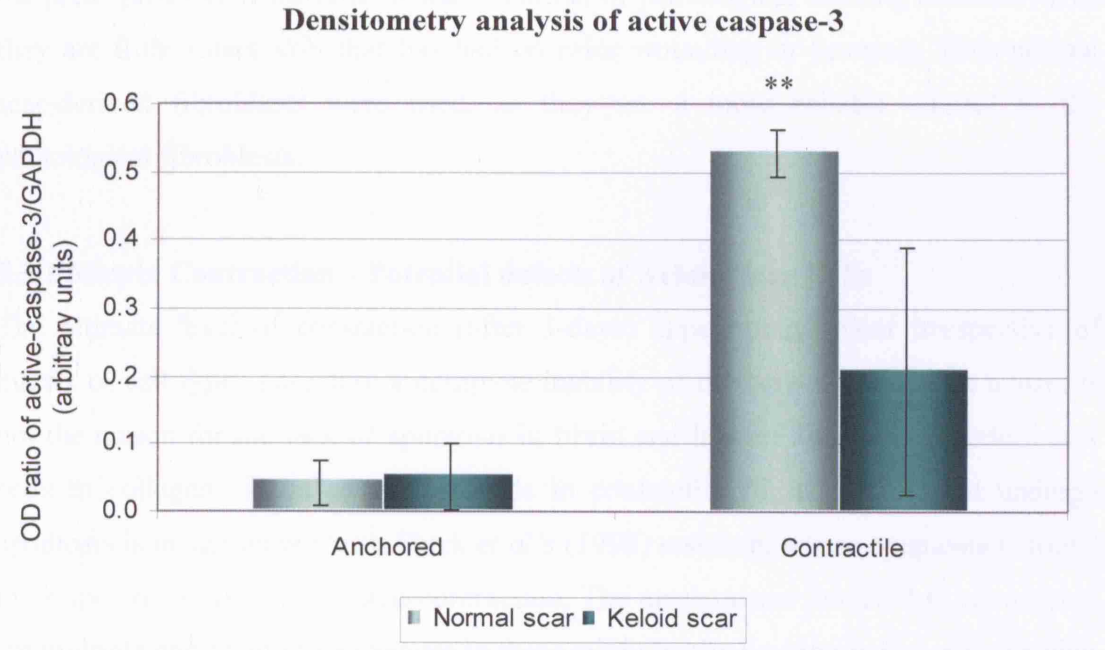


Figure 3.18B Densitometry analysis of western blots for caspase-3 with normal scar and keloid scar cells after 7-days of collagen matrix culture in anchored or contractile matrices. Expression levels of pro and active caspase-3 were analysed as a ratio to that of GAPDH. Results represent a mean of the experiment carried out on n=3 cell strains of each scar type, in triplicate. Error bars represent SEM. **P=<0.001 after T-test analysis.

3.3 Discussion

The major findings of this chapter are that normal scar fibroblasts but not keloid scar fibroblasts undergo apoptosis in response to the contraction of 3-D collagen gels but not fibrin gels. This apparent defect displayed by the pathological scar cells is specific to that induced in this wound healing model, since they show equal sensitivity to apoptosis induction by doxorubicin and ethanol.

3.3.1 Normal Scar Fibroblasts versus Dermal Fibroblasts

Results from normal scar fibroblasts are in agreement with Fluck *et al's* (1998) findings with dermal fibroblasts. This is important as these cells have gone through the process of wound healing and as such their behaviour may have been changed by this. Indeed, some researchers believe that scar cells may not originate from dermal fibroblasts, being a completely different cell type. The reason it was thought important to use normal scar-derived cells rather than dermal fibroblasts was because the predisposition of the latter towards normal or pathological scarring is unknown as they are from intact skin that has had no prior wounding or scarring. Thus normal scar-derived fibroblasts were used, as they are a more reliable control to the pathological fibroblasts.

3.3.2 Matrix Contraction – Potential defects of Keloid Scar Cells

The ultimate level of contraction (after 3-days) appears equivalent irrespective of matrix or cell type. Therefore a complete inability of the cells to contract a matrix is not the reason for the lack of apoptosis in fibrin and lack of apoptosis of keloid scar cells in collagen. The finding that cells in contractile fibrin gels do not undergo apoptosis is in agreement with Fluck *et al's* (1998) research, where apoptosis is found to be specific to that of collagen-contraction. The mechanisms involved in contraction are multiple and qualitative changes in these might be the limiting factor as to whether apoptosis is induced or not.

A more in-depth analysis of contraction induced by normal scar and keloid scar fibroblasts revealed significant differences. Normal scar fibroblasts contracted collagen matrices at twice the rate of that induced by keloid scar fibroblasts during the first 2-days after matrix release. This however was reversed by approximately day-3,

where keloid scar fibroblasts began to increase their rate of matrix contraction concomitantly with normal scar fibroblasts reducing theirs. Eventually, both scar types induced a similar degree of overall contraction. The rate of contraction detected with normal scar fibroblasts over the 5-days was in agreement with Stephens *et al* (2004); who found fibroblasts to initially contract collagen matrices at an increased rate immediately after the release of the matrix. The degree of matrix contraction then significantly slows down from day-2 onwards (Stephens *et al.,* 2004). The initial thrust of matrix contraction is thought to relate to the degree of tension generated within the matrix before its release and the elastic properties of the gel per se. The contraction that follows relies on cell-traction and additional cell-contraction (cell-shortening). Forces generated by cell-traction involve: cell motility, formation and stabilisation of focal adhesions, generation of contractile forces in the cytoskeleton and disassembly of adhesions and the regulation and interaction of cell surface receptors including β1- and β3-integrins and syndecan-4 with the extracellular matrix (Sethi *et al.*, 2002; Stephens *et al.,* 2004) In addition, cellular organisation and alignment of the extracellular matrix through the activation of ECM degrading enzymes is also a possible component of tractional-contraction/remodelling; since collagenase activity increases during collagen-contraction (Lambert *et al.*, 1992, 2001).

The initial slow rate of contraction seen with keloid scar fibroblasts may be due to a number of possible factors for instance: reduced build-up of residual cell-tension (elastic properties of the gel under tension), inadequate cell attachment with the extracellular matrix, disorganised cytoskeleton and inadequate cell spreading for the development of tractional forces compared to that which may occur within normal scar cell-seeded matrices. However, keloid scar fibroblasts do appear to be able to carry out the final phase of matrix contraction, which involves 'cellular-contraction' (section 3.1.3) which is suggested to occur after the initial release of residual matrix-tension and tractional remodelling. Thus, keloid scar fibroblasts may cause a reduced tractional force-driven contraction. This could be through a defect in the regulation of focal adhesions for stress-fibre formation, increased extracellular matrix stabilisation or aberrant integrin expression. Also remodelling and realignment of the extracellular matrix through cell-secreted proteases may be at fault. If there is an increase in the

rigidity of the extracellular matrix through excessive cross-linking or decreased degradation this may hinder gel contraction (Linge *et al.*, 2005). Results presented by Linge *et al* (2005) describe hypertrophic scar cells (another pathological cell type) as unable to respond to the effects of collagen contraction-induced apoptosis through excessive extracellular matrix cross-linking; specifically through the over-expression of tissue transglutaminase. Increased cross-linking has been associated with reduced disassembly/detachment of the established cell-matrix interactions thus hindering cell motility (Stephens *et al.*, 2004).

Investigation of the cellular morphology within contractile (stress-relaxed) collagen gels indicates that keloid scar cells are not responding in the same manner as normal scar fibroblasts to the dissipation of matrix tension on collagen-contraction. Keloid scars cells maintain a bipolar morphology suggesting that these cells do not 'relax' and are still under tension. It is possible that these cells are trying to pull on a 'rigid' gel, or there are differences in the expression of integrins or proteolytic enzymes involved in the remodelling of the ECM.

Morphological assessment of normal scar cells within contractile collagen gels revealed that the cells appeared to lose contact with the ECM and other cells, they appeared withered with many rounding-up; presumably undergoing apoptosis. It is possible that cells appear this way as they have simply been signalled to apoptose. Alternatively, the loose, withered appearance may reflect the dissipation of matrix tension and change in integrin expression; all known to occur during collagen-contraction, and thus the loss of survival signalling leading to apoptosis.

3.3.3 Possible Future Experimental Directions

A possible experiment to determine whether the appearance of cells within contractile collagen gels simply indicates the fact that cells have been signalled to apoptose or rather a reaction to the relaxed nature of the contractile gel, would be to treat normal scar cells in anchored collagen gels with the cytotoxic drug doxorubicin. If cells apoptose immediately without appearing loose and withered, this may suggest a possible mechanism of apoptosis induction, specific to that of collagen-contraction.

To determine more specifically the component of collagen-contraction that may be defective with keloid scar fibroblasts further investigations could be carried out into: initial matrix tension generated prior to gel release (using a culture-force monitor), cell motility and spreading, focal adhesion complex assembly, organisation of the cytoskeleton, cell surface receptor expression, ECM-degrading enzymes and ECM cross-linking. Experimental assessment could involve fluorescent confocal microscopy, transmission election microscopy, western blot analysis, RT-PCR, zymography and ECM degradation analysis.

3.3.4 Function of General Death Associated Proteins

Despite not responding to the apoptosis signals from collagen matrix contraction, keloid scar fibroblasts are able to respond to other apoptosis induction signals.

After treatment with the cytotoxic drug doxorubicin, western blot analysis determined that p53 stabilisation occurred in both normal scar- and keloid scar-derived fibroblasts. However, in non-treated control cells the basal expression of p53 was significantly ($p=0.030$) increased in keloid scar-derived fibroblasts. It is possible that irreparable cells are not being signalled to be destroyed via apoptosis in keloid scar derived fibroblasts as efficiently as normal scar derived fibroblasts; leaving increasing numbers of defective cells to be assessed via increasing levels of p53. Nevertheless, a similar rate of p53 stabilisation occurs in normal scar and keloid scar fibroblasts after 24hrs of drug treatment, with an approximate 4-fold increase in p53 levels. p53 mutations have previously been implicated in the aetiology of keloid scarring (Saed *et al.*, 1998). There have been studies on p53 mutations in keloids, however these are not written in English. The half-life of p53 is normally extremely short, the over-expression of p53 in keloid scar cells may be indicative of p53 mutations causing stabilisation of the protein. The p53 antibody used for this experiment does not detect differences between wild-type p53 and mutated p53. To investigate this further, fibroblasts derived from a large number of keloid scar strains should be analysed for mutations by DNA sequencing, by looking at exons that are most frequently mutated. To determine whether any mutations found could contribute to cell proliferation and apoptosis rates, an investigation of the effects of specific mutant forms of p53 could be studied in normal scar cells.

The expression of pro-caspase-3, in contrast to p53, is significantly higher in normal scar-derived fibroblasts in comparison to that of keloid scar fibroblasts. This is seen at both basal expression levels and after drug treatment. The expression of pro-caspase-3 in both scar types does not decrease on drug treatment concomitant with its activation, however this may be due to an up regulation in the expression of the protein itself. It is possible that the pro- form of caspase-3 is constantly expressed at high levels to allow a fast response in preparation for signalling a cell to apoptose. The production of the 17kDa fragment of active caspase-3 significantly increases in cells from both scar types on drug treatment. What is most noticeable is that the level of active caspase-3 is significantly higher in normal scar fibroblasts compared with keloid scar fibroblasts; although the fold-increase from untreated controls is relatively similar. The reduced levels of both the pro- and active caspase-3 may imply that keloid scar-derived fibroblasts produce reduced amounts of the protein through defective transcriptional regulation. It is possible that the reduced level of caspase-3 in keloid scar fibroblasts may play a role in their inability to respond to collagen remodelling-induced apoptosis. Although, this is perhaps argued against by the findings with the caspase-3-dependent cleavage product of activated PARP. PARP nevertheless, is a cleavage product of other caspases, where caspase-3 is found to have the highest affinity for PARP. It is therefore still possible that there is a fault with the caspase-3 protein in keloid scar-derived fibroblasts, where other effector caspases may act through a compensatory signal pathway.

As with caspase-3, activated PARP is expressed at significantly higher levels in normal scar fibroblasts in comparison to keloid scar fibroblasts, both at the basal level ($p=<0.001$) and after drug treatment ($p=0.041$). However, what is unexpected is the 20-fold increase in PARP activation on drug treatment seen in keloid scar fibroblast (which never exceeds that of normal scar) in comparison to just under a 2-fold increase with normal scar fibroblasts. The expression of the latent form of PARP nevertheless, is not significantly different between normal scar and keloid scar fibroblasts, this suggests that there is no fault with the production of this protein.

What is also clear to see is that caspase-3 activation peaks in both scar types at 8hrs then drops off, whereas the activation of PARP continually increases over the drug

time treatment. This may be due to the lag time of caspase-3 activation, where the cleaved caspase-3 fragment migrates into the cell nucleus to cleave PARP.

Assessment of caspase-3 activation after collagen matrix culture revealed that caspase-3 is specifically activated after collagen matrix contraction, but not in anchored collagen matrices. In addition, keloid scar fibroblasts failed to show a significant activation of caspase-3 after collagen matrix contraction, as detected with normal scar cells. This shows that collagen contraction-induced apoptosis does involve the activation of caspase-3. Generally however, the induction of apoptosis is not solely dependent on caspase-3 and can be independent, as extensively reviewed by Broker *et al* (2005), Kim *et al* (2005), and Kroemer and Martin (2005).

3.3.5 Summary

On reflection of the results gained in this chapter, there appears to be an intrinsic inability of keloid scar-derived fibroblasts to respond to the apoptosis-inducing effects of collagen matrix contraction. This is despite the ability of keloid scar fibroblasts to respond to the apoptosis cues induced by cytotoxic insult, which infers that at least some apoptosis pathways are intact. The possible mechanism of apoptosis induction that takes place during collagen gel contraction is investigated further in the next chapter (Chapter 4) along with the characterisation of possible faults with keloid scar-derived fibroblasts that may lead to an inability to respond to the apoptotic effects of collagen matrix contraction.

Chapter 4

Mechanisms of Collagen Contraction-Induced Apoptosis

4.1 Introduction

The results from Chapter 3 clearly demonstrate that keloid scar cells are specifically incapable of undergoing collagen contraction-induced apoptosis. Considering that this specific disability may underlie the whole pathology of this condition, it is important to gain a better understanding of the molecular basis of this defect. This chapter therefore concentrates on investigating the action of collagen contraction-induced apoptosis of normal scar cells together with attempting to define the defect exhibited by keloid scar cells. It is hoped that the latter will also serve to throw further light on which factors are important in normal wound healing-induced apoptosis.

4.1.1 What is Known About the Potential Apoptosis Mechanisms Involved in Wound Healing and 3-D Collagen Matrices?

Relatively few investigations have been carried out to unravel the underlying mechanisms involved in the induction of apoptosis during wound healing and collagen matrix contraction. There are many possible cues that might signal a cell to undergo apoptosis. For instance a simple change in cell shape can affect cell viability (Chen *et al.*, 1997). The actin cytoskeleton has been found to be an important regulator of cell shape and indeed cell survival. Mechanical tension generated within the actin cytoskeleton regulates diverse biological functions within the cell, being responsible for the anchoring and transport of different cellular components, and also plays a key role as a mediator of gene expression within the cytoplasm (Chicurel *et al.*, 1998). Research carried out by Dimmeler *et al* (1998) describes that fluid shear stress (a component of haemodynamic forces) stimulates the phosphorylation of the serine/threonine kinase Akt, which may contribute to the profound morphological and functional changes in human endothelial cells, potentially inhibiting cell apoptosis. In line with this research Chen *et al* (1999) discovered that fluid shear stress activates signal transduction pathways such as the Src homology and collagen protein (Shc), ERK and JNK in vascular endothelial cells. It is possible that stress exerted on mesenchymal cells during wound healing may have a similar affect on cell function, gene expression and intracellular signalling, which may inhibit or promote apoptosis.

Many studies demonstrate that cell-ECM interactions are critical during cell growth and differentiation; the molecular mechanisms linking these interactions with specific

cell functions are beginning to be unravelled. Cell-matrix interactions not only control the shape and orientation of cells but can also directly regulate diverse cell functions; such as proliferation, migration, differentiation and gene expression, and are known to play an important role throughout wound healing (Eckes *et al.*, 1999). The main cellular receptors for matrix proteins, integrins, have been found to synergise with other cellular stimulators such as growth factors (Brown and Hogg, 1996). It has been demonstrated that integrin binding and clustering activates intracellular signalling pathways, suggesting that these molecules are key cell surface signal (force) transducers (Damsky and Werb, 1992; Hynes, 1992; Schwartz and Ingber, 1994; Plopper *et al.*, 1995; Eckes *et al.*, 1999; Humphries *et al.*, 2004). Integrins however, do not possess intrinsic kinase activity; they must somehow associate with downstream signalling molecules. It is well known that integrins induce the formation of specialised cytoskeletal complexes or focal adhesion complexes (FACs) at the site of cell-ECM binding due to specific binding interactions between integrins, actin-associated molecules and actins (Burridge *et al.*, 1988; Plopper *et al.*, 1995; Humphries *et al.*, 2004). It has been suggested that this insoluble scaffolding in turn immobilises cell surface growth factor receptors as well as numerous chemical signalling molecules and brings them into close proximity within the FAC (Plopper *et al.*, 1995; Humphries *et al.*, 2004). This may facilitate cross talk between multiple signalling pathways. This signalling pathway should also be exquisitely sensitive to mechanical stresses because the FAC provides a path for mechanical signal transfer (Wang *et al.*, 1993).

Chen *et al* (1999) reported that the transduction of mechanical stimuli into chemical signals (Shc, ERK and JNK) leading to gene transcription is via integrins ($\alpha v\beta 3$, $\alpha 1\beta 1$ and $\alpha 5\beta 1$) in association with receptor tyrosine kinases, which together serve as mechanoreceptors.

It is clear from the research described above that integrins have an important role in transducing mechanical force into survival signals. It is possible therefore, that by removing the tension within the cells microenvironment (like that which occurs on collagen gel release) and/or preventing cell-matrix interactions, this may lead to anoikis (apoptosis through loss of cell contacts). Anoikis of endothelial and epithelial cell types is well established (Meredith *et al.*, 1993; Frisch and Francis, 1994; Re *et*

al., 1994; Ruoslahti and Reed, 1994; Boudreau *et al.*, 1995); however controversy still surrounds the ability of fibroblasts to undergo this particular form of apoptosis. Many authors report that fibroblasts and mesenchymal cells are resistant to anoikis cues, even after 50hrs of suspension culture (Meredith *et al.*, 1993, and references therein). This has been proposed to allow mesenchymal cells the ability to be migratory and motile, while in contrast, epithelial cells are tightly linked together and relatively static (Meredith *et al* 1993; Frisch and Francis, 1994; McGill *et al.*, 1997; Grossman, 2002). In contrast to this however, work carried out by Hadden and Henke (2000), propose that fibroblasts do undergo anoikis in response to fibronectin peptides, yet that this is delayed in comparison to epithelial and endothelial cells. Their work involved attempting to induce anoikis in adherent normal human lung fibroblasts, epithelial cells and endothelial cells using different fibronectin-derived peptides (RGD, CS-1 and FN-C/H-V). Unlike epithelial and endothelial cells, fibroblasts required the addition of all three peptides to respond. They found that fibroblasts underwent approximately 50% apoptosis after 3-4 days, whereas epithelial cells and endothelial cells responded to this cocktail of peptides or single peptides of RGD, CS-1 or FN-C/H-V much more quickly and easily, within 24hrs. They also repeated this experiment with non-adherent fibroblasts (cells in suspension) and found no additional apoptosis induction, thus they concluded that the apoptosis induced in fibroblasts is simply through disruption of fibroblast adhesion (anoikis). The explanation for the apparent contradictory results may simply be that mesenchymal cells derived from different origins differ in their sensitivity to anoikis. For example, studies claiming fibroblast resistance to anoikis were carried out on primary rat and mouse embryonic fibroblasts, as well as human cruciate ligament fibroblasts, however Hadden and Hanke (2000) used human lung fibroblasts.

Further investigation on the phenomenon of collagen-contraction dependant apoptosis by Niland *et al* (2001) has determined that it appears dependant on the action/expression of integrins (specifically $\alpha1\beta1$ and $\alpha2\beta1$), using both adhesion blocking antibody experiments and functional $\alpha2$ integrin transfection of $\alpha2$ null cells. Tian *et al* (2002) also found that ligation of the $\beta1$ integrin with anti-$\beta1$ integrin antibodies protected fibroblasts from apoptosis in contractile collagen matrices. The nature of the cell survival signal was found to be through PI3K acting through

Akt/protein kinase B. They showed that Akt phosphorylation decreased during collagen contraction, correlating with the onset of apoptosis. These results propose that the β1 integrin acts as a force mechanoreceptor, during collagen contraction, *directly or indirectly* leading to decreased Akt phosphorylation, and in turn apoptosis. The collagen contraction-induced apoptosis therefore appears separate from anoikis (the induction of apoptosis specific to blocking integrin binding). Both reports agree that collagen contraction-induced apoptosis seems to involve integrins. What is also important to note however, which was not discussed by Niland *et al* (2001) or Tian *et al* (2002) is the fact that integrin-mediated adhesion to ECM proteins regulates the expression of a vast array of genes, not simply the transduction of mechanical force which in turn may regulate kinase activity. For example, integrins can effect matrix metalloproteinase activity, pro-inflammatory and anti-inflammatory cytokines (Yamada and Miyamot, 1995), promoters of cell-cycle progression (Hansen *et al.*, 1994), modulators of cell death (Zhang *et al.*, 1995) as well as the activity of certain growth factor receptors (for example, β1-integrin can effect EGFR in 3-D breast cancer epithelial cell cultures, Wang *et al.*, 1998). Indeed, integrins are known to play an important role in collagen gel remodelling (a process induced during collagen contraction), with α1β1 controlling the down-regulation of collagen-1 expression and α2β1 controlling the induction of collagenases, MMP-1 (Langholz *et al.,* 1995) and MMP-2 (Seltzer *et al.*, 1994). It is possible therefore, that the connection with the β1 integrin complexes and collagen contraction-induced apoptosis, made by Niland *et al* (2001) and Tian *et al* (2002) is not as simple as previously thought, but rather an integral part of a much more complex signalling cascade, possibly involving distinct processes such as MMP degradation of the ECM.

4.1.2 Involvement of ECM Components in Collagen Contraction-Induced Apoptosis

A possible mechanism of collagen contraction-induced apoptosis, which has been relatively ignored, is the potential role of ECM breakdown products. This possibility is particularly intriguing given the recent findings of Buckley *et al* (1999), suggesting that small soluble RGD-motif-containing peptides that are potential breakdown products of ECM remodelling can induce apoptosis of fibroblasts, not only in the form of classical anoikis but also in a distinct fashion directly binding to and

activating caspase-3. In addition, apart from the mechanical differences between contractile and anchored collagen matrices the other major difference is with regards to their collagenase activity. During collagen gel contraction there is an up-regulation in collagenase transcription: MMP1 through $\alpha 2\beta 1$ (Lambert *et al.*, 1992; Langholz *et al.*, 1995), MMP-13 (Ravanti *et al.*, 1999b), MMP-2, -3, -9, and 13 (Lambert *et al.*, 2001) which is not observed within anchored collagen matrices. These differences would potentially lead to an increase in matrix breakdown products in contractile collagen gels, some of which would contain the RGD motif.

The RGD sequence was first discovered in fibronectin (FN) (Pierschbacher and Ruoslahti, 1984a) and was subsequently identified as a cell-attachment site found on many adhesion proteins (Pierschbacher and Ruoslahti, 1984b). It is known that when present within intact, ECM molecules, RGD (arg-gly-asp) motifs bind integrins and signal cell survival, preventing apoptosis induction. Prevention of cell adhesion through simple absence of a requisite substrate (Frisch and Francis, 1994) or blocking adhesion via small soluble RGD-containing peptides results in classical anoikis. For example, Vitale *et al* (1998) and Watanabe *et al* (1997) reported that this form of displacement provoked apoptosis of thyroid and endothelial cells. It has since been discovered that small soluble RGD-containing peptides are able to directly induce fibroblast apoptosis (Buckley *et al.* 1999; Perlot *et al.*, 2002). Initially using B and T lymphocytes, Buckley *et al* (1999) determined that RGD-containing peptides are internalised in an integrin-independent manner, accumulate in the cytosol of the cell, then bind to an RGD-binding site (DDM) found on pro-caspase-3, thus causing the auto-cleavage and activation of this central death protease. These authors also tested the effects of RGD-containing peptides on adherent cells (embryonic lung fibroblasts and breast carcinoma cell line MCF-7) as did Perlot *et al* (2002) (human osteoblasts, mouse MC-3T3-E1 cells and chick chondrocytes) and found that they were indeed potent inducers of apoptosis through capase-3 activation. Experiments performed by various research groups have again however, demonstrated a variation in the cellular apoptotic response. Results demonstrated by Hadden and Henke, (2000) discussed earlier, show that primary human lung fibroblasts were not sufficiently responsive to RGD-peptides alone, but required additional fibronectin peptides to undergo apoptosis. This again illustrates a variation in cell sensitivity to apoptosis inducing

effects, which may be linked to the source the fibroblastic cells were derived from or to other experimental differences.

4.1.3 The Possible Involvement of Matrix Metalloproteinases in Collagen Contraction-Induced Apoptosis

MMPs are the main family of proteases involved in the degradation and remodelling of the ECM during wound healing and have been implicated in 3-D collagen matrix contraction (Le *et al.*, 2002; Ravanti *et al.*, 1999a and 1999b; Scott *et al.*, 1998; Lambert *et al.*, 2001). Their action is required for the enzymatic breakdown of the ECM and therefore the production of breakdown products that could potentially induce apoptosis.

MMPs are a family of structurally-related zinc-dependent endopeptidases that are collectively capable of degrading essentially all ECM components. At present approximately 20 members of the MMP family have been characterised based on their structure and substrate specificity. The main subgroups include; collagenases, stromelysins, gelatinases and membrane-type MMPs (MT-MMPs) (Ravanti and Kahari, 2000). MMPs contain several conserved functional domains as illustrated in Figure 4.1.

The MMPs that are most likely to be involved in putative collagen remodelling-induced apoptosis are those specifically secreted by fibroblasts which include; MMP-1 (collagenase-1), MMP-2 (gelatinase A), MMP-3 (stromelysin-1) and MMP-13 (collagenase-3), all of which alongside other MMPs have been reviewed extensively by Ravanti and Kahari (2000), Stamenkovic (2000) and Visse and Nagase (2003), a summary of which is presented in Table 4.1.

These MMPs are also involved in homeostatic tissue turnover, metastatic growth, rapid remodelling and granulation tissue associated with wounds. These proteases are tightly controlled by their inhibitors (TIMPs), whose overall role is to form enzyme-inhibitor complexes (1:1 stoichiometry). TIMPs are involved in inhibiting tumourigenesis, cell invasion and metastasis (Mannello and Gazzanelli, 2001). They are also known to affect programmed cell death, with TIMP-1 being anti-apoptotic (Guedez *et al.*, 1998; Liu *et al.*, 2003) and TIMP-3 being pro-apoptotic (Bian *et al.*,

1996; Baker *et al.*, 1998, 1999; Ahonen *et al.*, 1998); a role that has been found to be independent of their MMP-inhibiting properties. The effects of TIMP-2 on apoptosis are more controversial with one report on melanoma cells indicating anti-apoptotic properties (Valente *et al.*, 1998), however other publications on breast epithelial and B cells found no effect of TIMP-2 on apoptosis (Guedez *et al.*, 1998; Liu *et al.*, 2003). The roles of the four TIMPs have been reviewed by Mannello and Gazzanelli, 2001 and Visse and Nagase, 2003, a summary of which is presented in Table 4.2.

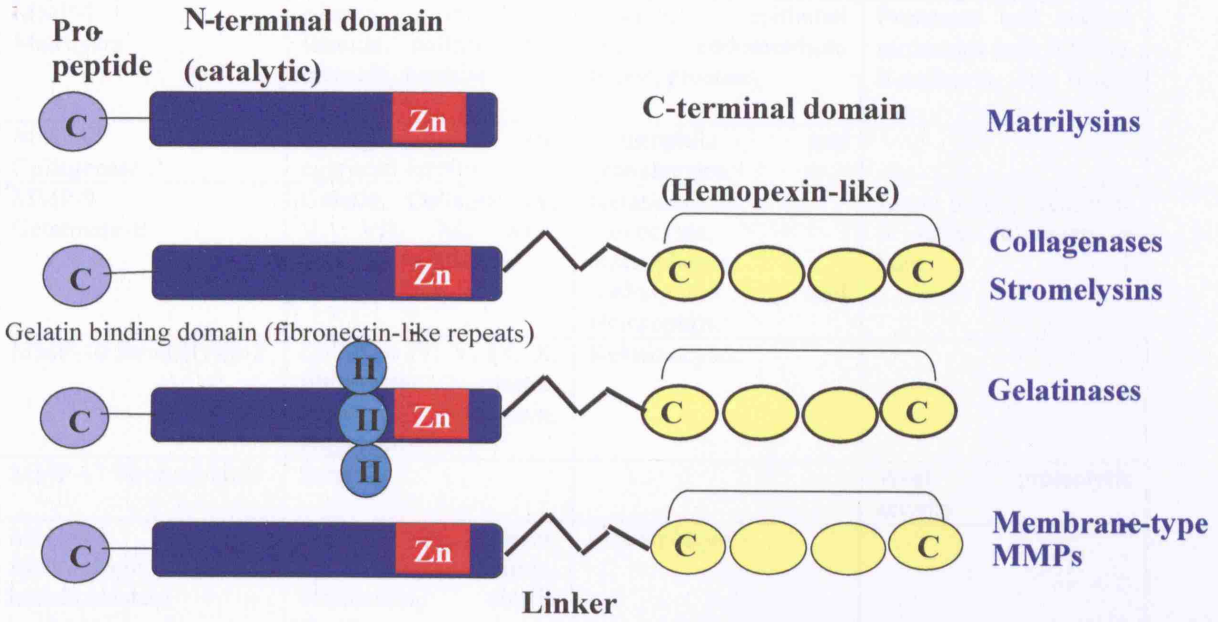


Figure 4.1. Illustration of the conserved sequences present on MMPs. A signal peptide directs the proenzyme for secretion (~ 80aa); the cysteine residue forms a covalent bond with the zinc fragment to maintain the latency of the pro-MMP. The catalytic domain contains the highly conserved zinc domain. The proline-rich hinge region links the catalytic domain to the C-terminal hemopexin domain, which determines the substrate specificity of MMPs and mediates interaction with tissue inhibitors of MMPs (TIMPs) (adapted from Ravanti and Kahari, 2000).

Table 4.1 Matrix metalloproteinases and their actions

MMP	Substrates	Produced by	When seen
MMP-1 Collagenase-1	Collagen I, II, III, VII, VIII, X, aggregan, serpins.	Keratinocytes and dermal fibroblasts, macrophages, chondrocytes.	Produced on acute and chronic wounding.
MMP-2 Gelatinase-A	Gelatin, Collagen I, IV, V, VII, X, fibronectin, tenascin, fibrillin.	Keratinocytes, smooth muscle cells and dermal fibroblasts	Linked to metastatic growth and associated with daily tissue remodelling.
MMP-3 Stromelysin-1	Collagen IV, V, IX, X, fibronectin, elastin, gelatin, laminin, aggrecan, fibrillin.	Keratinocytes and dermal fibroblasts.	Expressed on chronic wounding, abundant in granulation tissue
MMP-7 Matrilysin	Elastin, fibronectin, laminin, collagen IV, tenascin, versican.	Gladular epithelial cells, endometrium, breast, prostate.	Processes cell surface molecules incl: TNF-α, E-cadherin, Fas ligand etc.
MMP-8 Collagenase-2	Collagen I, II, III, aggrecan serpins.	Neutrophils and granulocytes	
MMP-9 Gelatinase-B	Gelatin, Collagen IV, V, VII, XI, XIV, elastin, fibrillin.	Keratinocytes, monocytes, macrophages, eosinophils and Neutrophils.	Acute injury, present 4-5 days following a burn.
MMP-10 Stromelysin-2	Collagen IV, V, IX, X, fibronectin, elastin, gelatin, laminin, Aggrecan.	Keratinocytes.	
MMP-11 Stromelysin-3	Serpins.		Weak proteolytic activity.
MMP-12 Macrophage metalloelastase	Collagen IV, gelatin, fibronectin, laminin, vitronectin, elastin fibrillin.	Macrophages.	
MMP-13 Collagenase-3	Collagen I, II, III, IV, IX, X, XIV, gelatin, fibronectin, laminin, aggrecan, fibrillin, serpins.	Dermal fibroblasts in 3-D culture. Cancer cells, gingival cells.	Seen in chronic dermal ulcers not acute. Associated with rapid healing.
MMP-14 MT1-MMP	Collagen I, II, III, gelatin, laminin, vitronectin, aggrecan, tenascin, fibrillin, fibrin.		Important in activating pro-MMP-2, role in angiogenesis.
MMP-15 MT2-MMP	Fibronectin, laminin, aggrecan, tenascin.		Important in activating pro-MMP-2
MMP-16 MT3-MMP	Collagen III, fibronectin, gelatin, casein, proteoglycans.		Important in activating pro-MMP-2.

Table 4.2 Functions of TIMPs

TIMP	Expression	Biological activities
TIMP-1	Soluble form, responds to stimuli	Forms specific complexes with pro-MMP-9, potentiates cell growth, over-expression reduces tumour growth, anti-apoptotic activities. Does not inhibit ADAMs (a disintegrin and metalloproteinase) and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motif)
TIMP-2	Soluble form, constitutively expressed	Effective inhibitors of MT-MMPs, forms a complex that is important in cell-surface activation of pro-MMP-2, potentiates cell growth, over-expression reduces tumour growth, Does not inhibit ADAMs and ADAMTSs
TIMP-3	Insoluble bound to ECM	Effective inhibitors of MT-MMPs and TNF-α converting enzyme, over-expression reduces tumour growth, has pro-apoptotic activities. Inhibits ADAMs and ADAMTSs
TIMP-4	Acts in a tissue specific fashion on ECM	Binds to C-terminal domain of pro-MMP-2. Does not inhibit ADAMs and ADAMTSs

4.1.4 The Involvement of Caspase-3

As previously stated, Buckley *et al*, (1999) suggested that RGD peptides activate the apoptotic cascade by directly activating pro-caspase-3. The primary processing site between the p12 and p17 subunits in pro-caspase-3 lies next to a DDM sequence, which is a potential RDG-binding site (Nicholson *et al*., 1995). Buckley *et al*, (1999) proposed that RGD-peptides exerted their effects by binding to this site, inducing conformational changes that lead to auto-processing of caspase-3. This was confirmed using the breast carcinoma cell line (MCF-7), which has a functional deletion of caspase-3 (Buckley *et al*., 1999). RGD-peptides were unable to induce apoptosis of wild-type MCF-7 cells; however, caspase-3 transfectants underwent rapid cell death with RGD-peptides. Caspase-3 cleavage was confirmed by immunoblotting and inhibition assays (Buckley *et al*., 1999). Supporting this possibility are more recent reports that RGD-containing peptides induce apoptosis in osteoblasts (Perlot *et al*., 2002) and in leukaemia HL-60 cells (Anuradha *et al*., 2000) through caspase-3. Despite evidence pointing towards the possible involvement of caspase-3, it is feasible that alternative effector caspases may be directly involved in collagen contraction-induced apoptosis, for instance caspase-8, -7 or -6.

4.1.5 Aim

It is clear that multiple factors have the potential to be involved in collagen contraction-induced apoptosis including purely mechanical changes (cell-stress, tension, cell-shape etc.) and/or biochemical changes (cell surface receptors such as integrins and growth factor receptors, matrix breakdown effectors or products).

This chapter therefore concentrates on attempting to determine the mode of apoptosis induction that takes place during collagen contraction, by investigating the involvement of both mechanical (cell-tension, cell-contacts) and biochemical (proteolytic remodelling of the ECM and the effect of the proteolytic breakdown products) apoptosis cues. In addition, this chapter attempts to understand why keloid scar-derived fibroblasts are unable to respond to these cues.

4.2 Results

4.2.1 Mechanical Cues of Collagen Contraction Induced Apoptosis

4.2.1.1 Is Collagen Contraction Induced Apoptosis Dependant on the Dissipation of Cell Tension and Cell Contacts?

Experiments were carried out to investigate whether the dissipation of cell contacts and cell tension are alone sufficient to cause the apoptosis seen during collagen contraction. Factors to be tested were added to the *in vitro* gel model in the culture media. The potent toxin Cytochalasin D causes the collapse of the actin cytoskeleton, resulting in the dissipation of cell contacts leading to cells rounding up and thus reduction of tension within the cells (Flanagan and Lin, 1980; Niland *et al.*, 2001; Ailenberg and Silverman, 2003). The effect of Cytochalasin D was therefore investigated on normal scar-derived fibroblasts seeded within 3-D collagen gels. Figure 4.2A shows the percentage of cell death induced after 7-days of collagen matrix culture with or without the addition of Cytochalasin D. Contractile collagen matrices were used as a positive control. Cytochalasin D was added to anchored matrices as an alternative method of removing cell contacts and tension. Results shown in Figure 4.2A clearly depict a significant induction of apoptosis (P=0.01) in day-7 contractile matrices compared to day-7 anchored matrices cultured in minimal growth media alone. However, the addition of Cytochalasin D to the minimal growth media at day-4 (instead of releasing the matrix to contract) had no significant effect

on live cell number by day-7. This was despite the fact that cells were rounded up in appearance having lost their cell contacts along with the elimination of cell tension (Figure 4.2B).

Despite reducing the tension within the cell itself by collapsing the actin cytoskeleton no apoptosis was induced. However, these cells are still surrounded by ECM proteins that have not been broken down (collagenase activity is minimal in anchored collagen gels) which could still be supplying survival signals to the rounded up cells. For this reason, a classical anoikis experiment was carried out, where cells maintained in serum free media rounded up (under non-adherent culture which lacked a substrate that would allow cell attachment and spreading) in the absence of ECM signalling for 3-days (time-course of contraction). Cells were harvested daily, reseeded into tissue culture-grade dishes to allow cell adhesion and monitored for signs of cell death (non-attachment of cells) after 24hrs. Results from this experiment are shown in Figure 4.3 where, after 72hrs (checked daily at 24, 48 and 72hrs) of non-adherent culture in serum free conditions no cell death was induced in either normal scar- or keloid scar-derived fibroblasts. Under light microscopy (Figure 4.3) all cells were able to re-adhere to tissue culture plates (as judged by the absence of rounded up, floating cells) and appeared healthy. It is clear from Figure 4.3 that keloid scar cells are more clumped together when adhering to the tissue culture-grade dishes compared to normal scar cells. It is possible that keloid scar cells are more reliant on cell-cell contacts for survival than normal scar cells. This finding however, provides evidence that fibroblasts, whether they are of a normal phenotype (normal scar) or a pathological phenotype (keloid scar) do not appear to undergo anoikis even after 72hrs of non-adherent culture.

Together these results suggest that simply removing cell tension and cell contacts from the collagen matrix environment the cells are embedded within is not sufficient to induce scar fibroblast apoptosis. Therefore collagen contraction-induced apoptosis is not simply due to anoikis; the rounding up of cells or the potential removal of survival signals from the ECM by enzymatic breakdown during the remodelling that takes place in contractile collagen gels. This is not to say that scar fibroblasts are completely resistant to the effects of anoikis as discussed in section 4.1, but rather, they do not undergo anoikis over the time-course of collagen gel contraction.

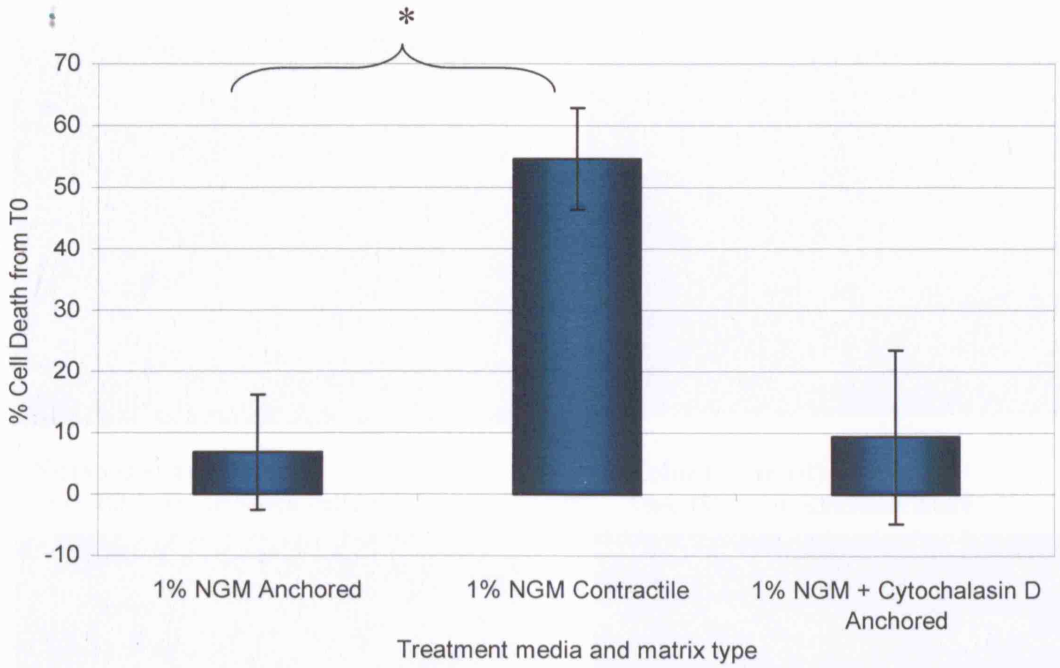


Figure 4.2A Collagen matrices were seeded with normal scar-derived fibroblasts and maintained in 1% NGM. Matrices were retained anchored for the full 7-days, or for contractile gels for 4-days prior to releasing the matrix for 3-days. Test matrices were treated with 20μM Cytochalasin D (Sigma) at day-4 instead of releasing the matrices. Cells were harvested at day-0 and day-7, where viable cell number was assessed. Results displayed represent the percentage of cell death at day-7 from day-0. This experiment was carried out with n=3 normal scar cell strains in triplicate. *P=<0.05 using T-test analysis.

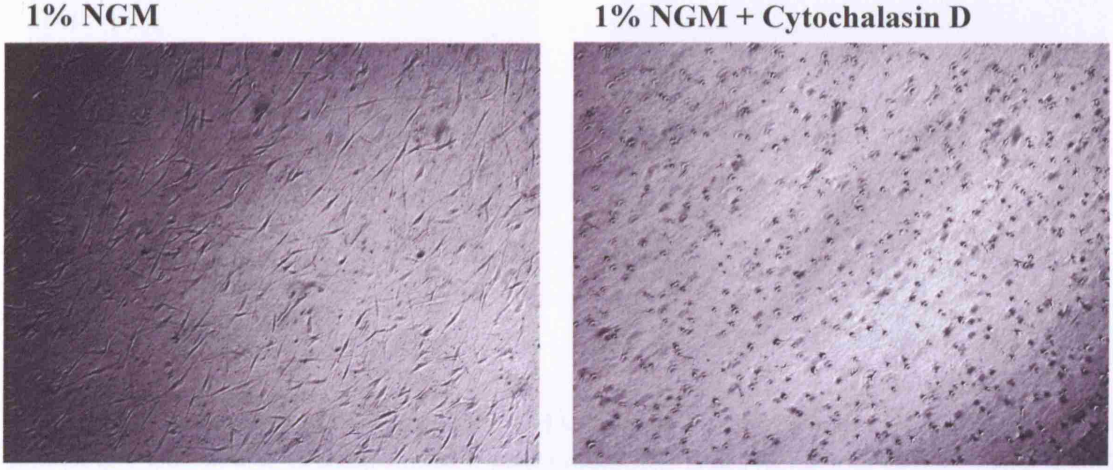


Figure 4.2B Typical morphological appearance of normal scar fibroblasts seeded into anchored collagen matrices at day-7 of gel culture under a light microscope. On refreshing the 1% NGM at day-4, the fibroblasts were either cultured for a further 3-days in 1% NGM or 1% NGM + 20μM Cytochalasin D. This experiment was carried out with n=3 normal scar cell strains in triplicate. (x100 Mag).

4.2.1.2 Is Collagen Contraction Induced Apoptosis Dependant on More than Simply Mechanistic Cues?

To further determine the apoptotic effects a contractile collagen matrix has upon normal scar cells, an experiment was carried out where day-7 contractile or anchored collagen matrices were homogenised and added to monolayer cultures of normal scar fibroblasts. The aim of this experiment was to determine if biochemical changes within contractile gels induced apoptosis of monolayer cells. Note that the results given in this section are part of a larger experiment, which is presented in its entirety in section 4.2.2.1, but are presented separately here for reasons of clarity.

Figure 4.4 shows the effect of various homogenised gel solutions including, acellular collagen gels (acellular collagen control), cell-containing collagen gels: normal scar cell-conditioned anchored gels (anchored control), normal scar cell-conditioned contractile gels (contractile collagen test) and normal scar cell-conditioned anchored gels that contain apoptotic cells induced by pre-treatment with ethanol (apoptotic cell control), on monolayer cultured normal scar cells after 24hrs. All gels were washed before homogenisation (particularly important to remove all traces of ethanol) before adding to cell monolayers. Aprotinin was added to all homogenates to prevent any possible non-specific death through proteolysis.

Only the homogenised collagen gel that had been conditioned and contracted by normal scar cells was able to induce cell death in normal scar cell monolayers ($p=0.003$). This phenomenon was not therefore due to the presence of cells per se, as no cell death occurred with the anchored control. In addition, the absence of monolayer apoptosis induction by the apoptotic cell control demonstrates that the apoptosis induced in cell monolayers is not simply due to the action of proteases released from apoptotic cells within the gels during homogenisation.

These results indicate that contractile-collagen gels are conditioned by normal scar cells in such a way as to produce biochemical cues of apoptosis. Although the initiating event of collagen contraction-induced apoptosis is undoubtedly mechanical these results clearly demonstrate a biochemical component to the induction of collagen contraction/remodelling-induced apoptosis. How intrinsically linked these two events are, is unknown. However, the fact that keloid scar cells contract collagen

matrices but do not undergo apoptosis may imply that, the biochemical changes involved in the induction of apoptosis are not an absolute requirement for successful contraction.

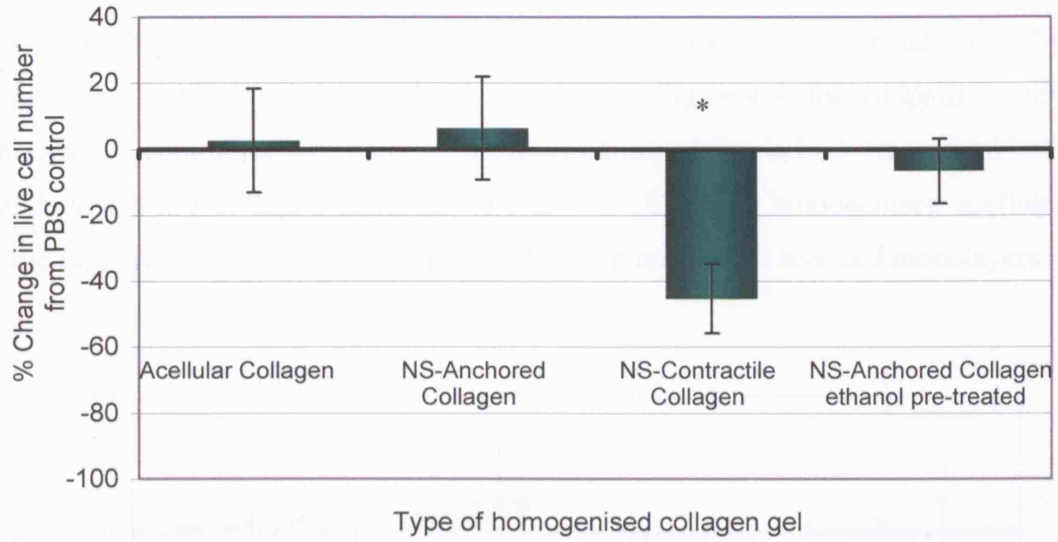


Figure 4.4. The effects of homogenised anchored and contractile collagen matrices on monolayer cultures of normal scar cells. Collagen matrices were conditioned by normal scar cells (NS) for 7-days prior to being homogenised in PBS. Homogenised acellular collagen gels were used as a control in addition to cell-conditioned anchored collagen that had previously been treated with 10% ethanol to induce cell apoptosis. Cell monolayers were cultured on collagen type I (10μg/ml) coated 6-well plates in SFM for 48hrs prior to adding relevant homogenised gel solution. After 24hrs of treatment, live cell number was assessed by Trypan Blue exclusion. This result represents the percentage change in live cell number from PBS only control. The experiment was carried out with n=4 normal scar cell strains in triplicate. T-test analysis compared the percentage change in live cell number after homogenised gel treatment to that of PBS treated cells. *P=<0.05.

4.2.2 Biochemical Cues of Collagen Contraction-Induced Apoptosis

4.2.2.1 Are Keloid Scar Cells able to Produce these Biochemical Apoptosis Cues or Respond to those Produced by Normal Scar Cells?

It has already been determined that keloid scar cells are able to contract a collagen matrix eventually to an equivalent degree to normal scar cells (Chapter 3); however they do not undergo apoptosis. It is possible that this is because keloid scar cells fail to either produce or respond to the biochemical cues of apoptosis.

To investigate this possibility the effects of homogenised contractile collagen gels previously conditioned by either keloid scar or normal scar cells were examined on both normal scar and keloid scar cell monolayer cultures.

Figure 4.5 plots data from both the effects of homogenised anchored (anchored control) and contractile collagen (contractile collagen test) matrices conditioned by normal scar fibroblasts (as previously shown alone in Figure 4.4) for comparison with homogenised contractile collagen matrices conditioned by keloid scar fibroblasts (keloid contractile collagen test), as well as the effects of homogenised acellular collagen gels (acellular control) on both normal scar and keloid scar cell monolayers.

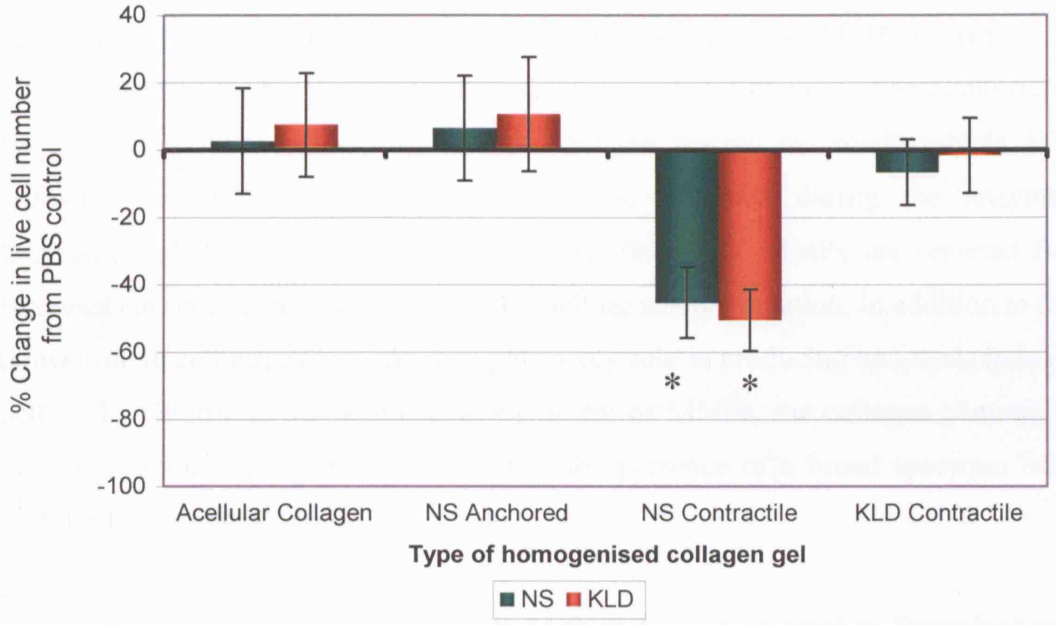


Figure 4.5 The effect of cell conditioned collagen gel homogenates. Normal scar (NS) or keloid scar cells (KLD) were cultured in anchored or contractile gels for 7-days in 1% NGM. Matrices were then homogenised in PBS. This gel solution was then aliquoted onto monolayer cultures of keloid scar or normal scar fibroblasts. Monolayer cells were plated on collagen (10μg/ml) coated 6-well plates in SFM for 48hrs prior to adding relevant homogenised gel solution. After 24hrs of treatment, live cell number was assessed by Trypan Blue exclusion. This result represents the mean of n=3 normal scar and keloid scar strains carried out in triplicate. Error bars represent SD. T-test analysis compared the percentage change in live cell number after homogenised gel treatment to that of PBS treated cells. *P=<0.05.

Figure 4.5 demonstrates that anchored control homogenates had no significant effect on the live cell number of cultured monolayers of either scar type. Normal scar contractile collagen test homogenates did induce cell death significantly, by approximately 50%, in both normal scar ($p=0.003$) and keloid scar ($p=0.008$) cell monolayer cultures. However, keloid contractile collagen test homogenates had no significant effect on the live cell number of either normal scar or keloid scar cell monolayer cultures. Results from Figure 4.5 suggest that keloid scar fibroblasts, during the contraction of collagen gels do not effectively condition the gel to produce the right conditions to induce apoptosis; either through a failure to produce the correct apoptosis cues or because of producing anti-apoptotic signals (survival signals) which counteract the apoptotic cues.

4.2.2.2 Does Collagen Contraction Induced Apoptosis Require MMP Activity?

It is possible that the biochemical apoptosis cues produced by normal scar cells during the contraction and remodelling of the collagen matrix are small soluble ECM fragments or RGD-motif containing peptides, liberated during the enzymatic degradation of the ECM (as discussed in section 4.1.2). MMPs are secreted from fibroblasts in order to assist in matrix remodelling and degradation, in addition to their known role in cell migration, and may play a key role in producing apoptosis inducing factors. To determine the potential involvement of MMPs, the collagen contraction-induced apoptosis assay was performed in the presence of a broad spectrum MMP inhibitor (Ilomastat, GM6001).

Figure 4.6A plots the percentage of cell death at day-7 calculated as the reduction in total live cell number from that at T0. Figure 4.6A shows that the addition of Ilomastat (at day-4, concomitant with releasing the collagen gels) to contractile collagen matrices seeded with normal scar fibroblasts completely inhibited the significant ($p=0.024$) induction of apoptosis seen at day-7 in contractile collagen gels cultured in 1% NGM only. In addition, Ilomastat had no significant effect on the live cell number in anchored collagen matrices compared to those cultured in anchored matrices with 1% NGM alone. The effect of Ilomastat was therefore determined to be specific to inhibiting cell death rather than inducing cell proliferation. On assessing the overall degree of contraction induced by the normal scar fibroblasts (Figure 4.6B), there was a small reduction in the degree of gel contraction seen with Ilomastat-

treated matrices, however this was not significant (Table 4.3). Figure 4.6A and B further demonstrates that the degree of matrix contraction as a whole is not specifically related to the induction of apoptosis.

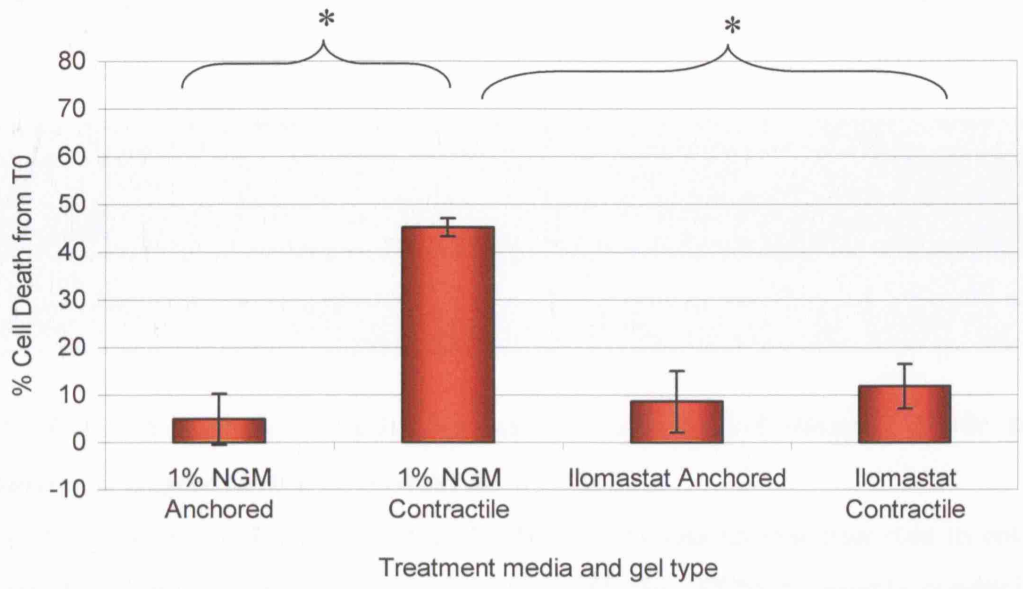


Figure 4.6A Normal scar fibroblasts were embedded into collagen matrices, which were maintained anchored for 7-days, or 4-days prior to releasing the gels and allowing them to contract for 3-days. At specific time points (0-, 4-, and 7-days) viable cell counts were performed. Results displayed represent the percentage of cell death induced at day-7 from T0. Cells were cultured in 1% NGM. At day-4, on refreshing the culture media, 5μM of Ilomastat (GM6001-broad spectrum MMP inhibitor) was added to the media of the appropriate gels. This experiment was carried out with n=4 normal scar cell strains in triplicate. T-test analysis compared the percentage of cell death induced between each different gel treatment at day 7. *P=<0.05.

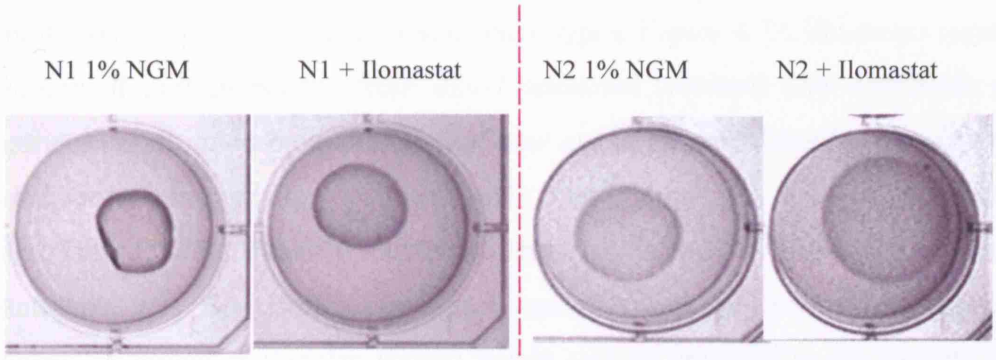


Figure 4.6B Typical degree of gel contraction induced by normal scar fibroblasts by day-7 of collagen gel culture. Cells were cultured in either 1% NGM throughout the 7-day culture period or received 5μM of Ilomastat to the 1% NGM at day-4 of the culture time course (on gel release). This Figure shows two normal scar cell strains (N1 + N2). This experiment was carried out with n=4 normal scar cell strains in triplicate.

Table 4.3 The mean degree of contraction induced by normal scar fibroblasts embedded in collagen matrices, at day-7 of gel culture. An MMP inhibitor (Ilomastat) was assessed by addition to the medium at day-4 of gel culture (on gel release). At day-7, the matrix circumference was measured in reference to the perimeter of the tissue culture well (original circumference of the gel). The difference given is the mean of n=4 normal scar cell strains, carried out in triplicate. 1% NGM was also assessed as a control.

Treatment media	Mean circumference of gel – periphery of well (arbitrary units)	Standard deviation between of samples (arbitrary units)	P-value (T-test)
1% NGM	209.48	±27.88	
Ilomastat	203.70	±51.80	0.815

4.2.2.3 Do Keloid Scar Cells Degrade a Contractile-Collagen Matrix to an Equivalent Degree to that of Normal Scar Cells?

Results from Figure 4.6 suggest that MMP activity has an essential role in collagen contraction-induced apoptosis, possibly by producing ECM fragments conducive to apoptosis. It is feasible that keloid scar-derived fibroblasts do not produce adequate signals to degrade the ECM to a comparable degree to that of normal scar-derived fibroblasts during collagen contraction. In this manner keloid scar cells may not produce sufficient extracellular breakdown products capable of inducing apoptosis.

Silver staining of SDS-PAGE gels was used as a sensitive method of detecting polypeptides present in collagen gels that have been conditioned and remodelled by fibroblasts derived from the two scar phenotypes. Figure 4.7A illustrates typical silver staining of protein lysates from day-7 anchored (control) and contractile collagen matrices conditioned by either normal scar or keloid scar fibroblasts (matrices where lysed containing cells). Collagen gels conditioned by normal scar fibroblasts demonstrated significantly different banding patterns between anchored and contractile gels. Specifically, contractile gel preparations revealed a reduction in the density of higher molecular weight bands and an increase in the density of lower molecular weight bands (asterix – Figure 4.7A). This was not detected in contractile matrices conditioned by keloid scar cells where the banding patterns were remarkably similar to that of anchored gels (of both keloid and normal scar) (Figure 4.7A). Figure 4.7B plots band density against molecular weight along each of the lanes seen in Figure 4.7A. It is very obvious from the graph (Figure 4.7B) that in gels conditioned

by normal scar fibroblasts there are more polypeptides of <50kDa in contractile matrices compared to anchored matrices and less polypeptides between 50 and 150kDa. This pattern was highly reproducible for each of the 4 strains tested. This potentially suggests that protein degradation is taking place within the contractile matrices, leading to the loss of large molecular weight proteins and the accumulation of smaller peptides. When studying Figure 4.7B, assessing the banding pattern from keloid scar cell-conditioned anchored collagen matrices, there is a marked similarity with that of normal scar cell-conditioned anchored matrices. Interestingly however, the polypeptide banding pattern is almost identical between the anchored and contractile matrices conditioned by keloid scar cells; where protein peaks appear at similar molecular weight positions over most of the whole range (Figure 4.7B). Collagen gel contraction by keloid scar cells is not accompanied by a decrease in larger molecular weight (50-150kDa) polypeptides nor is their any apparent accumulation of polypeptides of <50kDa.

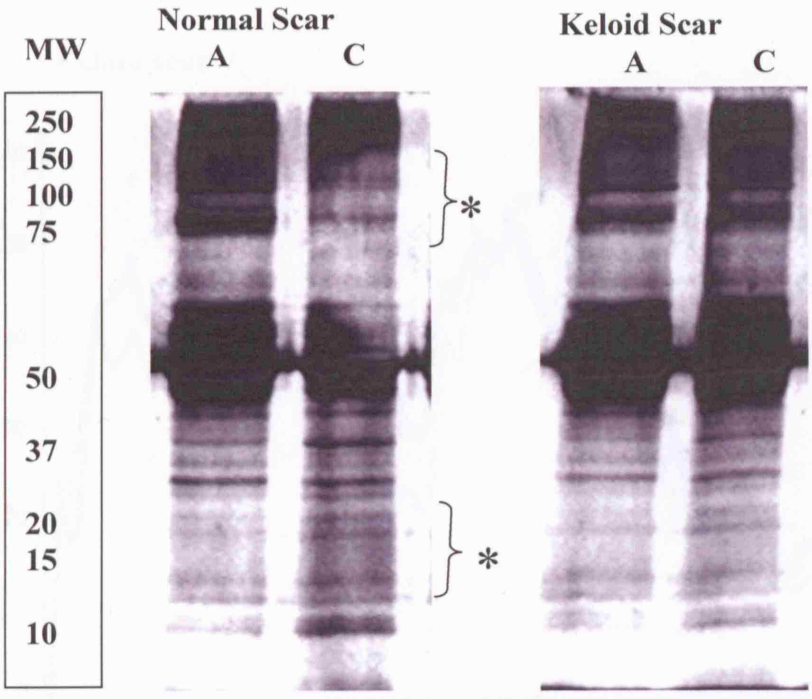


Figure 4.7A. Typical silver staining of anchored (A) and contractile (C) collagen gel lysates at day-7. Normal scar or keloid scar fibroblasts were cultured in collagen matrices for 7-days maintained in 1% NGM. The cells within the collagen matrices were lysed in SDS lysis buffer and run on a 10% SDS gel. This experiment was repeated three times with n=4 normal scar and keloid scar cell strains. Major changes in band decreases between anchored and contractile gel preparations are marked with an asterix. (MW = KDa).

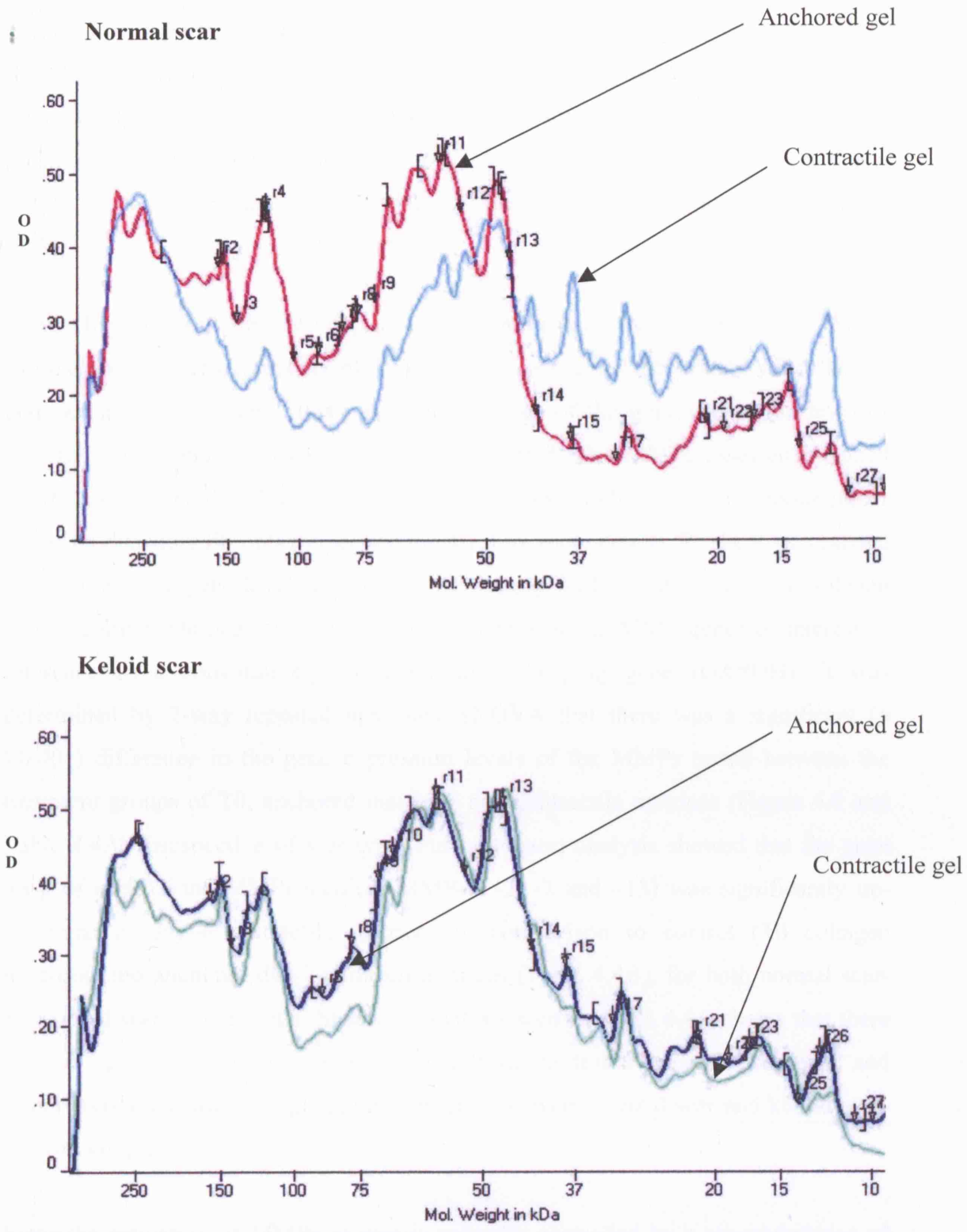


Figure 4.7B Representative densitometry analysis of silver stained collagen gel lysates (n=4 cell strains per scar type) analysing protein band size (kDa). The x-axis represents the optical density of each protein band. Protein lysates were from day-7 anchored and contractile matrices conditioned by normal scar or keloid scar fibroblasts. Brackets and arrows represent numbered peaks.

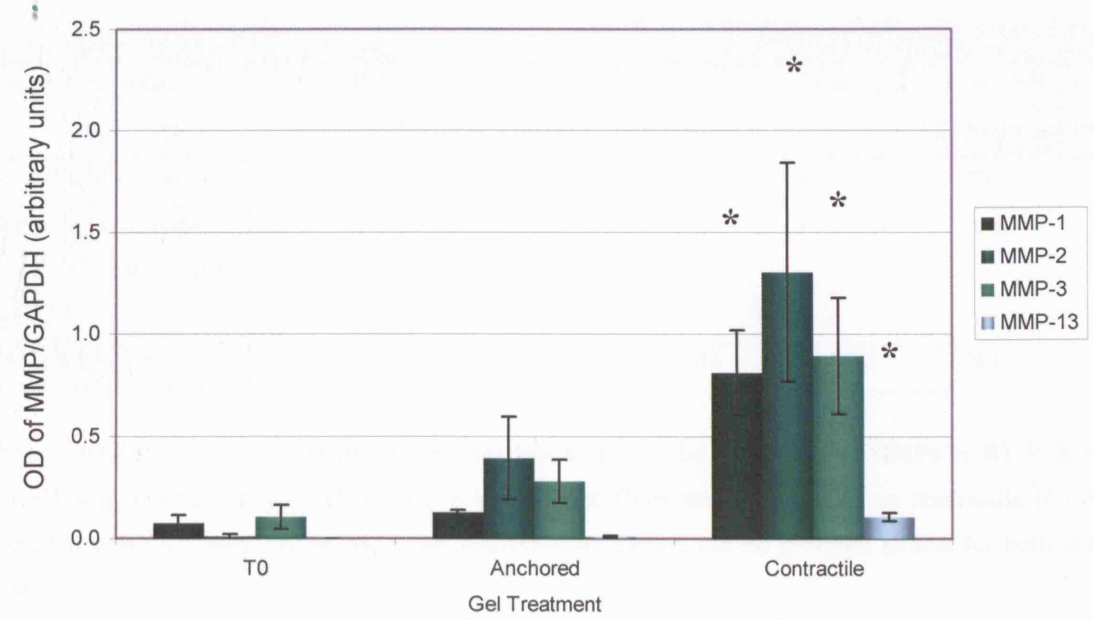
4.2.2.4 Do Keloid Scar Cells have an Aberrant Gene Expression of MMPs and TIMPs in Comparison with that of Normal Scar Cells?

Results from Figure 4.7 provide evidence that keloid scar cells are unable to condition a contractile collagen gel in such a way as to produce polypeptide species that are potentially small breakdown products of ECM fragments (theoretically, biochemical apoptosis cues). This may be the result of aberrant enzymatic degradation of the ECM.

It was hypothesised that keloid scar-derived fibroblasts may have reduced MMP expression or over-expression of TIMPs in response to the effects of collagen-contraction. To investigate this possibility analysis of the gene expression level of specific MMPs and TIMPs were therefore assessed. The MMPs assessed included MMP-1, -2, -3 and –13. Each of these MMPs is known to be involved in tissue repair and is produced by fibroblasts (see section 4.1.3 for more detail). RT-PCR was carried out to assess the gene levels of these MMPs at day-0 (T0) and at day-7 of collagen matrix culture. On analysing the expression levels of the MMP genes of interest in reference to a constitutively expressed house-keeping gene (GAPDH), it was determined by 2-way repeated measures ANOVA that there was a significant ($p > 0.001$) difference in the gene expression levels of the MMPs tested between the treatment groups of T0, anchored matrices, and contractile matrices (Figure 4.8 and Table 4.4A); irrespective of scar type. Further, T-test analysis showed that the gene level of each of the MMPs studied (MMP-1, -2, -3 and –13) was significantly up-regulated in day-7 contractile matrices in comparison to control (T0 collagen matrices) and anchored day-7 collagen matrices (Table 4.4B), for both normal scar- and keloid scar-derived cells. Statistical analysis seen in Table 4.4A shows that there was no significant variation between the cell strains tested for each scar type, and importantly there was no significant difference between normal scar and keloid scar-derived samples.

Since the activation of MMPs *in vivo* is naturally controlled by a counterbalance of TIMP expression, RT-PCR of TIMP-1, -2, -3 and -4 was carried out to assess whether keloid scar-derived fibroblasts exhibit an aberrant expression of these inhibitors.

Normal scar



Keloid scar

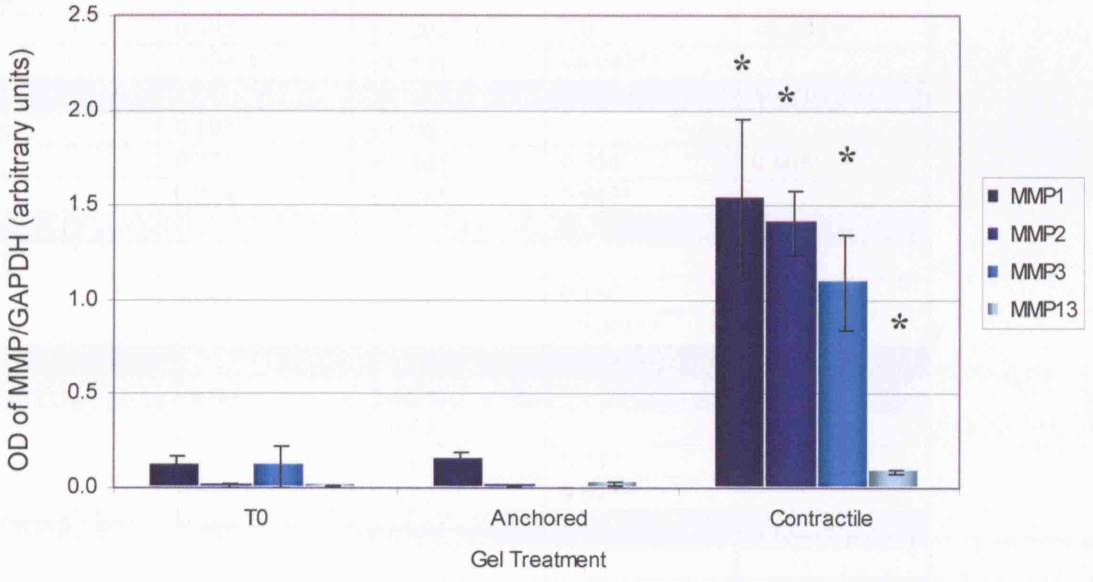


Figure 4.8 Densitometry analysis of RT-PCR results. Analysis of the RNA expression levels of MMP-1, -2, -3 and -13 from day-0 (T0) and day-7 anchored and contractile collagen matrices seeded with normal scar or keloid scar fibroblasts. Cells were cultured in 1% NGM. Results are presented as the expression of the gene of interest as a ratio to that of GAPDH. Results are the mean of n=8 each of normal scar and keloid scar cell strains. Error bars represent SEM. T-test analysis compared gene expression at T0 vs that at day-7 in anchored or contractile gels. *P=<0.05.

Table 4.4A Statistical analysis of the raw data from normal scar (NS) and keloid scar (KLD) MMP RT-PCR densitometry results, using 2-way repeated measures ANOVA (C=contractile, A=Anchored gels).

2-Way Repeated Measures ANOVA					
Genes Analysed	**MMP-1**	**MMP-2**	**MMP-3**	**MMP-13**	**Statistical Significance**
Between Samples	P= 0.114	P= 0.63	P=0.857	P=0.314	**No**
Between treatment groups (T0, C, A) for NS & KLD	**P>0.001****	**P>0.001****	**P>0.001****	**P>0.001****	**Yes**
NS vs KLD	P=0.148	P=0.593	P=0.385	P=0.68	**No**

Table 4.4B Results from T-test analysis of raw data assessing the expression of MMPs by RT-PCR in normal scar and keloid scar cell seeded collagen gels. Cells were maintained in contractile (C) or anchored gels (A), with 1% NGM. T-test analysis was carried out on n=8 cell strains for both scar types.

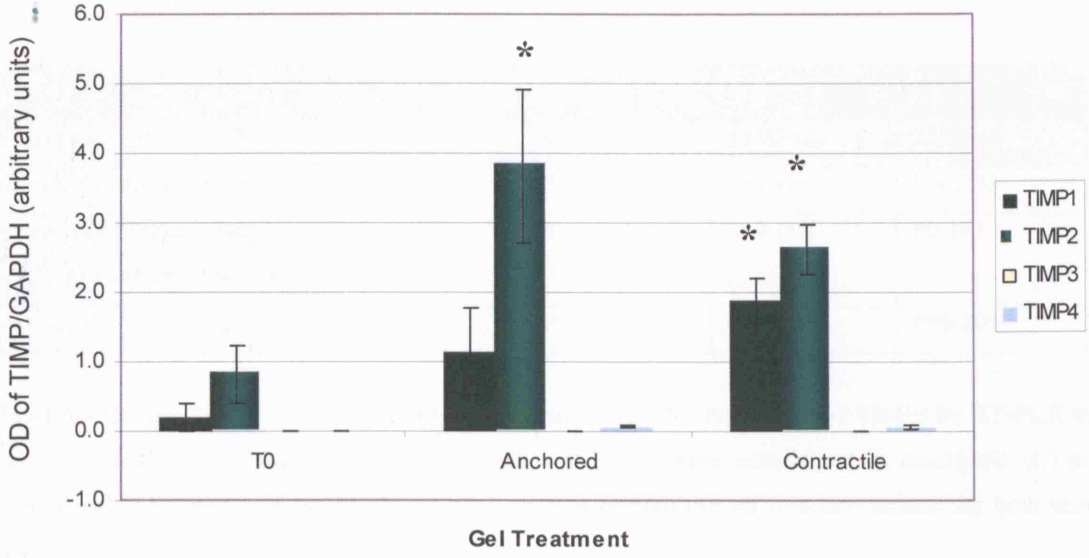
Normal Scar			P-value	
MMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.076	± 0.039		
A	0.129	± 0.012	0.656	**0.017***
C	0.811	± 0.208	**0.015***	
MMP-2			**Vs T0**	**Vs C**
T0	0.011	± 0.01		
A	0.392	± 0.203	1.0	**<0.001****
C	1.304	± 0.538	**<0.001****	
MMP-3			**Vs T0**	**Vs C**
T0	0.107	± 0.06		
A	0.281	± 0.105	0.356	**0.005***
C	0.894	± 0.285	**0.013***	
MMP-13			**Vs T0**	**Vs C**
T0	0.00	± 0.00		
A	0.009	± 0.006	0.160	**0.003***
C	0.107	± 0.02	**<0.001****	
Keloid Scar			**P-value**	
MMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.118	± 0.05		
A	0.150	± 0.04	0.242	**0.017***
C	1.535	± 0.42	**0.013***	
MMP-2	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.012	± 0.01		
A	0.009	± 0.005	0.205	**0.035***
C	1.408	± 0.165	**0.011***	
MMP-3	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.112	± 0.11		
A	0.000	± 0	0.207	**0.046***
C	1.094	± 0.255	**0.038***	
MMP-13	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.008	± 0.005		
A	0.025	± 0.011	0.334	**0.013***
C	0.081	± 0.011	**0.007***	

Figure 4.9 illustrates the gene expression level of the TIMPs assessed in both normal scar and keloid scar cell-seeded matrices, with reference to the house-keeping gene GAPDH. Two-way repeated measures ANOVA (Table 4.5A) were used to examine the data from Figure 4.9, and showed that there was a significant difference in all the TIMPs assessed apart from TIMP-4, between gel treatments (T0, anchored and contractile).

Further data analysis using t-tests (Table 4.5B) determined that TIMP-1 expression by normal scar cells appeared to be significantly ($p=0.021$) increased in contractile day-7 matrices in comparison to control (T0) matrices (Table 4.5B). Keloid scar cells also expressed significantly increased levels of TIMP-1 in contractile gels compared to both that expressed at T0 ($p=0.012$) and that expressed in anchored gels ($p=0.019$) (Figure 4.9 and Table 4.5B). There appeared to be no significant difference in the expression level of TIMP-1 between normal scar- and keloid scar-derived fibroblasts (Table 4.5A). TIMP-2 however, did show a significant difference ($p=0.009$) in gene expression levels between the two scar types (Table 4.5A).

As with TIMP-1, TIMP-2 was expressed at significantly ($p=0.003$) different levels between the gel treatments (Table 4.5A). Specifically, normal scar cells appeared to express a significant increase in TIMP-2 in both contractile and anchored day-7 gels in comparison to T0 gels ($p=0.008$ and 0.009, respectively) (Table 4.5B). The expression of TIMP-2 was not significantly different between anchored and contractile day-7 matrices ($p=0.385$). Keloid scar fibroblasts however, showed only a significant increase in TIMP-2 gene expression in contractile day-7 gels compared to both T0 gels ($p=0.027$) and anchored day-7 gels ($p=0.004$). Whereas keloid scar cells expressed only a very low level of TIMP-2 in anchored day-7, which was comparable to control (T0) matrices (Figure 4.9). Importantly, it appears that the levels of TIMP-2 expressed by normal scar cells in anchored and contractile day-7 matrices was not significantly different to that expressed by keloid scar cells in contractile day-7 matrices ($p=0.514$ and 0.670, respectively).

Normal scar



Keloid scar

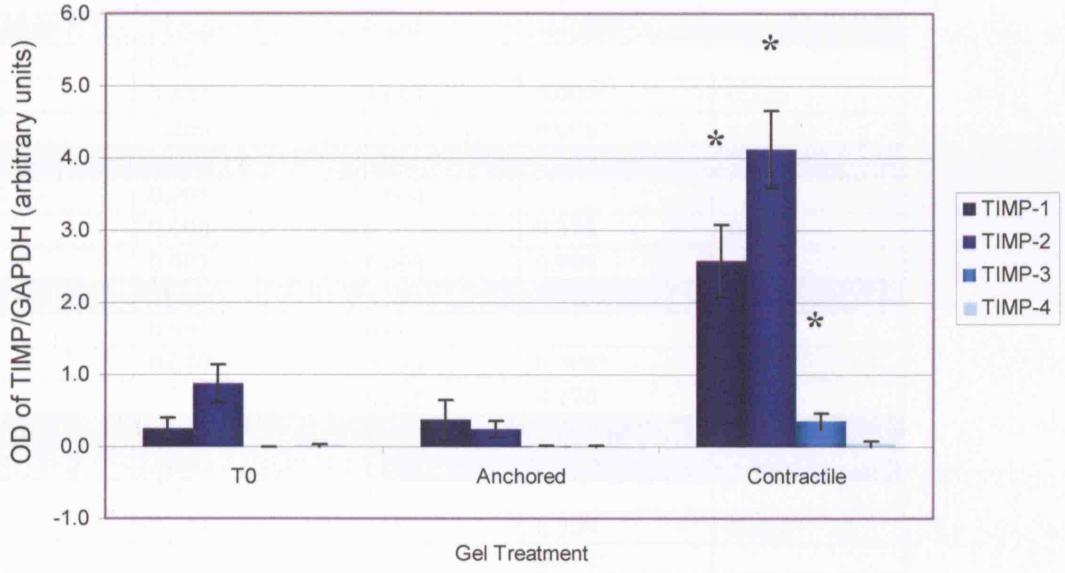


Figure 4.9 Densitometry analysis of RT-PCR results. Analysis of the RNA expression levels of TIMP-1, -2, -3 and -4 from day-0 and day-7 anchored and contractile collagen matrices seeded with normal scar or keloid scar fibroblasts. Cells were cultured in 1% NGM. Results are presented as the expression of the gene of interest as a ratio to that of GAPDH. Results are the mean of n=8 each of normal scar and keloid scar cell strains. Error bars represent SEM. T-test analysis compared gene expression at T0 vs that at day-7 in anchored or contractile gels. *P=<0.05.

Table 4.5A Statistical analysis of the raw data from normal scar (NS) and keloid scar (KLD) TIMP RT-PCR densitometry results, using 2-way repeated measures ANOVA (C=contractile, A=Anchored gels).

2-Way Repeated Measures ANOVA				
Genes Analysed	**TIMP-1**	**TIMP-2**	**TIMP-3**	**TIMP-4**
Between Samples	P= 0.988	P= 0.069	**P=0.006**	P=0.293
Between treatment groups (T0, C, A) for both NS & KLD	**P>0.001**	**P=0.003**	**P=0.002**	P0.197
NS vs KLD	P=0.203	**P=0.009**	**P=0.002**	P=0.201

Table 4.5B Results from T-test analysis of raw data assessing the expression of TIMPs by RT-PCR in normal scar and keloid scar cell seeded collagen gels. Cells were maintained in contractile (C) or anchored gels (A), with 1% NGM. T-test analysis was carried out on n=8 cell strains for both scar types.

Normal Scar			P-value	
TIMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.201	± 0.192		
A	1.147	± 0.633	0.227	0.365
C	1.876	± 0.33	0.021	
TIMP-2	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.820	± 0.412		
A	3.824	± 1.097	**0.009***	0.223
C	2.260	± 0.354	**0.008***	
TIMP-3	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.004	± 0.004		
A	0.000	± 0	0.374	0.374
C	0.003	± 0.003	0.904	
TIMP-4	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.001	± 0.001		
A	0.070	± 0.014	**0.005***	0.08
C	0.049	± 0.025	0.120	
Keloid Scar			**P-value**	
TIMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.256	0.152		
A	0.373	0.275	0.729	**0.019***
C	2.582	0.5	**0.012***	
TIMP-2	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.873	± 0.263		
A	0.247	± 0.116	0.098	**0.004***
C	4.122	± 0.535	**0.027***	
TIMP-3	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.000	± 0		
A	0.010	± 0.005	0.123	**0.035***
C	0.356	± 0.109	**0.032***	
TIMP-4	**Mean**	**± SEM**	**Vs T0**	**Vs C**
T0	0.016	± 0.018		
A	0.009	± 0.009	0.374	0.374
C	0.040	± 0.04	1.0	

Figure 4.9 shows TIMP-3 to be expressed at very low levels in both scar types. Although, Table 4.5A shows that there is a significant difference in the gene expression levels of TIMP-3 between gel treatments (p=0.002) and between scar types (p=0.002), there is also a significant variation (p=0.006) between the cell strains assessed for each scar type. This therefore makes it impossible to accurately interpret changes in gene expression levels of TIMP-3 between normal scar and keloid scar fibroblasts. The expression of TIMP-3 nevertheless, is significantly increased in keloid scar cell-seeded contractile gels versus T0 gels (p=0.032) and anchored gels (p=0.035). The expression of TIMP-3 by keloid scar cells in contractile day-7 gels in also significantly increased compared to that expressed by normal scar cells in contractile day-7 gels (p=0.033). TIMP-4 on the other hand, showed no significant change in expression levels between the gel treatments or between scar types and remained at a relatively low expression level over the 7-day matrix culture period (Figure 4.9 and Table 4.5A and B).

The results from Figures 4.8, 4.9 and Tables 4.4 and 4.5 appear to indicate that the gene expression of MMPs is specifically increased on matrix contraction, which is in agreement with their possible involvement in the apoptosis that takes place during collagen-contraction. However, these results also demonstrate that keloid scar and normal scar fibroblasts in contractile gels generally demonstrate equivalent expression of the MMP and TIMP genes examined, with the possible exception of an increase in TIMP-3 by keloid scar cells. The results from RT-PCR analysis does not provide conclusive evidence however, a more accurate technique for detecting mRNA expression levels could be carried out, for example quantitative RT-PCR.

Since the gene expression levels of these ECM cleavage enzymes appears relatively normal in keloid scar-derived fibroblasts, investigations were carried out to determine the involvement of specific proteases in producing the biochemical apoptosis cues, and to go on to compare their activity in keloid scar versus normal scar fibroblasts.

4.2.2.5 Does MMP-2 have a Specific Role in Collagen Contraction-Induced Apoptosis?

As mentioned previously in section 4.1.3, MMP-2 is a gelatinase; this protease acts to degrade small collagen proteins previously cleaved by collagenases (including MMP-1 and -13) into very small gelatin fragments. If the biochemical cues of apoptosis are indeed small soluble RGD-motif containing peptides or ECM fragments, then the protease most likely responsible for their release is a gelatinase. Although other MMPs secreted by fibroblasts are suggested to exhibit gelatinase activity (Table 4.1), MMP-2 is known to be the major gelatinase secreted by fibroblasts. MMP-2 is also known to be involved in daily tissue remodelling and furthermore, is up-regulated during wound repair (considered further in section 4.1.3). As such therefore, MMP-2 is an ideal candidate for the production of small soluble RGD-peptides during collagen-contraction.

In order to investigate whether MMP-2 alone could affect collagen contraction induced apoptosis a specific inhibitor of MMP-2 (Oleoyl-N-hydroxylamide) was used to treat the *in vitro* collagen gel model. Results seen in Figure 4.10A show that MMP-2 does indeed seem to have a role in the induction of apoptosis that takes place during matrix contraction. As with Ilomastat (broad spectrum inhibitor) the MMP-2 inhibitor was added at day-4, concurrent with collagen gel release, and completely abrogated the significant ($P=0.004$) induction of apoptosis that occurred on matrix contraction in minimal growth medium alone (Figure 4.10A). Moreover, as with Ilomastat (Figure 4.6), the MMP-2-specific inhibitor had no significant effect on live cell number in anchored collagen matrices compared to cells cultured in anchored collagen matrices in minimal growth medium alone. On assessing matrix contraction, the specific inhibitor of MMP-2 had no significant effect on the overall degree of gel contraction at day-7 of matrix culture (Figure 4.10B and Table 4.6).

In order to further confirm the involvement of MMP-2, recombinant TIMP-2 protein was added to the collagen gel model, by addition to the minimal growth medium at day-4.

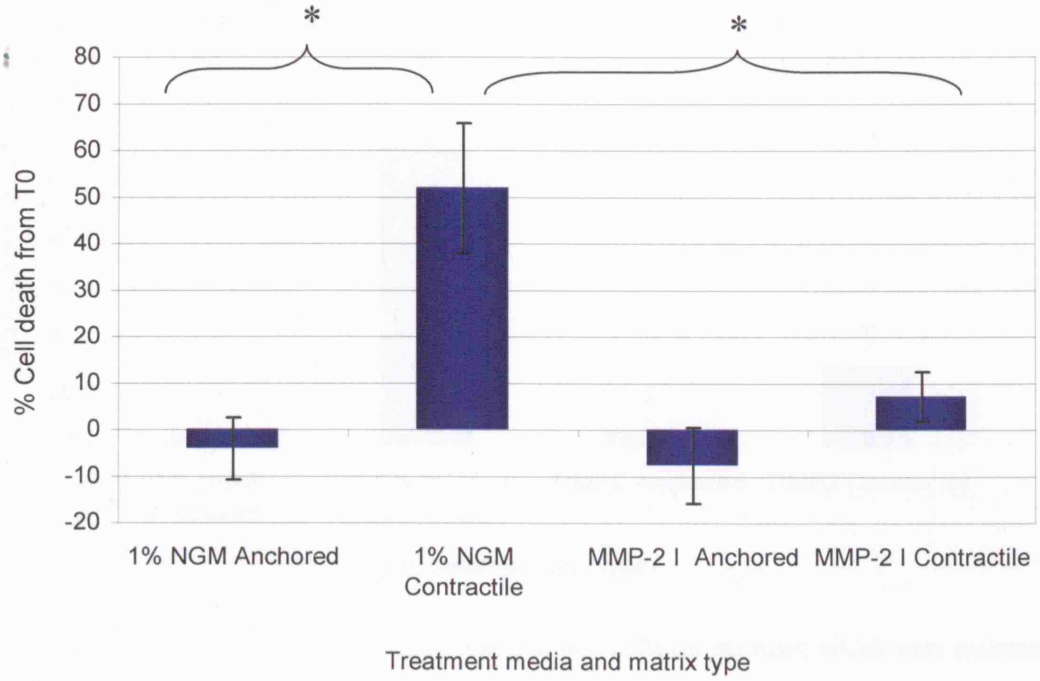


Figure 4.10A Normal scar fibroblasts were embedded into collagen matrices, which were maintained anchored for 7-days or 4-days prior to releasing gels to contract for 3-days. Viable cell counts were performed at day-0, -4 and -7. Results displayed represent the percentage of cell death at day-7 from T0. Cells were cultured in 1% NGM. At day-4, on refreshing the culture media, 10μM of a specific matrix metalloproteinase-2 inhibitor (MMP-2I) was added to the media of appropriate collagen gels. The results plotted are the mean of n=4 normal scar cell strains carried out in triplicate. T-test analysis compared the percentage of cell death induced at day-7 between each gel treatment. *P=<0.05.

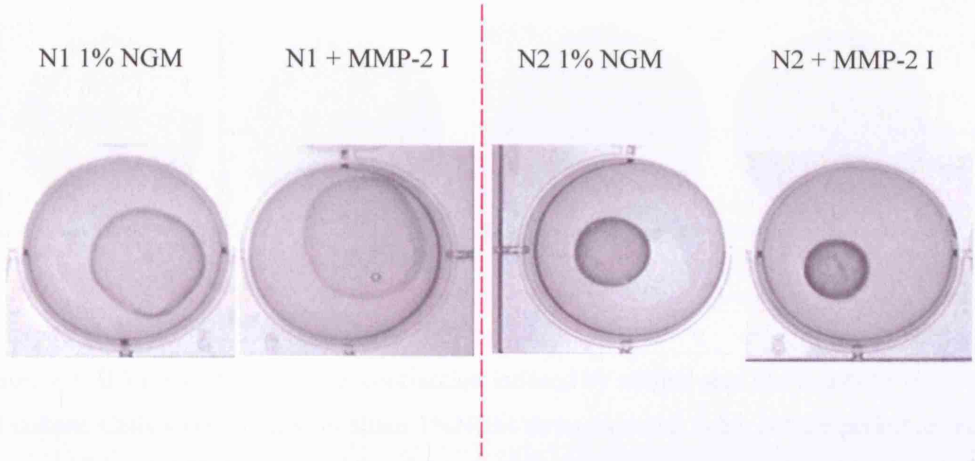


Figure 4.10B Typical degree of gel contraction induced by normal scar fibroblasts by day-7 of collagen gel culture. Cells were cultured in either 1%NGM throughout the 7-day culture period or received 10μM of a specific MMP-2 inhibitor in the media (+MMP-2I) from day-4 of the culture time course (on gel release). This Figure represents two normal scar strains (N1 and N2). This experiment was carried out with n=4 normal scar cell strains in triplicate.

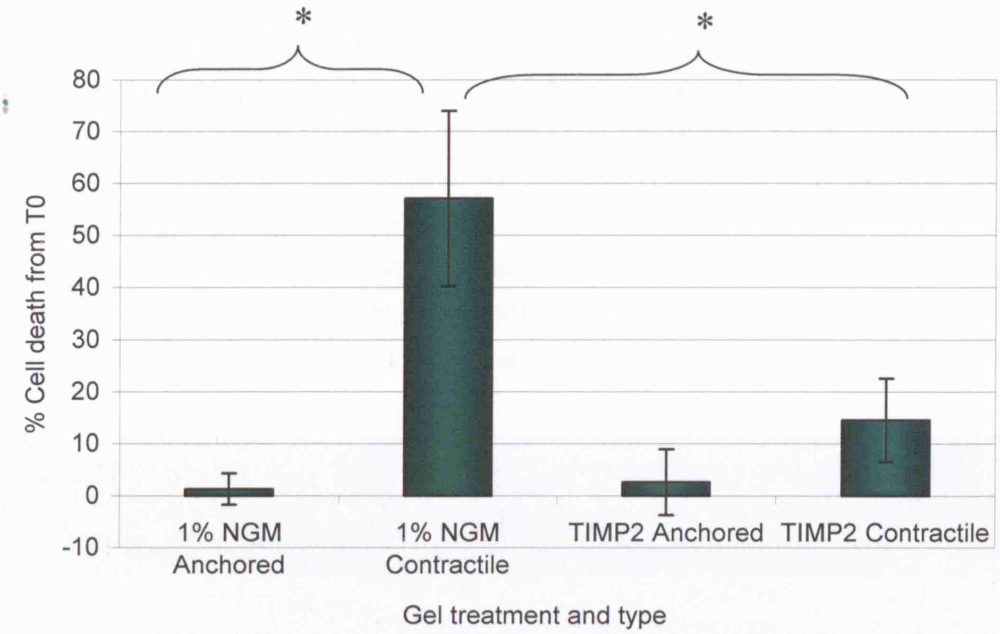


Figure 4.11A Normal scar fibroblasts were embedded into collagen matrices, which were maintained anchored for 7-days or 4-days prior to releasing gels to contract for 3-days. Viable cell numbers was assessed at day-0, -4 and -7. Results displayed represent the percentage of cell death at day-7 from T0. Cells were culture in 1% NGM. At day-4, on refreshing the culture media, 2nM of human recombinant TIMP-2 was added to the media of the appropriate collagen gels. The result plotted are the mean of n=4 normal scar cell strains carried out in triplicate. T-test analysis compared the percentage of cell death induced at day-7 between each gel treatment. *P=<0.05.

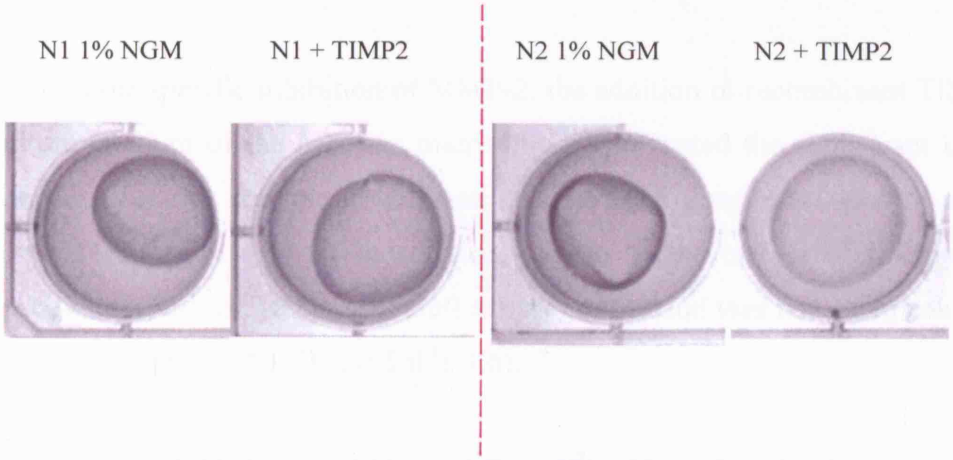


Figure 4.11B Typical degree of gel contraction induced by normal scar fibroblasts by day-7 of collagen gel culture. Cells were cultured in either 1%NGM throughout the 7-day culture period or received 2nM of recombinant TIMP-2 in the media at day-4 of the culture time course (on gel release). This Figure represents two normal scar strains. This experiment was carried out with n=4 normal scar cell strains in triplicate.

Table 4.6 The mean degree of contraction induced by normal scar fibroblasts embedded in collagen matrices, at day-7 of gel culture. Inhibitors of MMP activation (specific-inhibitor of MMP-2 and recombinant TIMP-2) were assessed by the addition to 1% NGM at day-4 of gel culture (on gel release). At day-7, the matrix circumference was measured in reference to the perimeter of the tissue culture well (original circumference of the gel). The difference given is the mean of is n=4 normal scar cell strains, carried out in triplicate. For each factor tested control media (1% NGM) was also tested. Experiments with the specific-inhibitor of MMP-2 and recombinant TIMP-2 were carried out at separate times therefore data for 1% NGM medium alone is different for each.

Treatment media	Mean circumference of gel – periphery of well (arbitrary units)	Standard deviation between of samples (arbitrary units)	P-value (T-test)
1% NGM	290.427	± 28.586	
MMP-2 inhibitor (10μM)	278.723	± 65.816	0.698
1% NGM	181.197	± 17.353	
Recombinant TIMP-2 (2nM)	152.212	± 23.487	0.977

TIMP-2 is a naturally occurring inhibitor of MMPs with a high affinity for MMP-2. In addition, the use of TIMP-2 specifically inhibits MMP activity (Visse and Nagase, 2003) whereas the pharmaceutical MMP inhibitors can also inhibit other enzymes such as ADAMs (A Disintegrin Associated Metalloproteinase) (Kang *et al.*, 2004).

As with the specific inhibition of MMP-2, the addition of recombinant TIMP-2 to the culture medium of the collagen matrix model prevented the significant induction of apoptosis seen in contractile collagen gels cultured in minimal growth media alone ($p=0.011$) (Figure 4.11A). In addition, similar to the effects of Ilomastat and the specific inhibitor of MMP-2, overall matrix contraction was not significantly affected by TIMP-2 (Figure 4.11B and Table 4.6).

4.2.2.6 Do Keloid Scar and Normal Scar Fibroblasts Exhibit Equivalent Levels of Active MMP-2?

Results presented in this chapter so far appear to suggest that MMPs and specifically MMP-2, may have a role in the induction of apoptosis that occurs during collagen matrix contraction. However, the gene expression levels of the MMPs studied are not significantly different between normal scar- and keloid scar-derived fibroblasts when

in contractile gels; nor are the expression levels of TIMP-1 and -2. This therefore leaves the question as to why keloid scar cells are not responding to the apoptotic effects of collagen matrix contraction if the genes supposedly involved in matrix remodelling are being expressed at an equivalent level to normal scar cells. One possibility is that the proteins are not either translated or becoming activated and are therefore ineffective. Zymography was therefore carried out to determine the activation of MMP-2 (Figure 4.12B, C). Conditioned culture media from anchored and contractile collagen gels at day-7 was assessed for MMP-2 activity, by measuring the expression of active MMP-2 as a ratio to that of the pro-MMP-2 expressed. Samples were standardised by seeding equal cell numbers into the gels at day-0, and since the experiments were performed under quiescent conditions (1% NGM), the conditioned media at day-7 was from equivalent cell numbers. Figure 4.12B and C clearly demonstrates that MMP-2 is significantly ($P<0.001$) activated in contractile collagen matrices in comparison to anchored collagen matrices. This was true for the conditioned medium from both normal scar- and keloid scar-seeded matrices. However, the level of active MMP-2 is significantly ($P<0.001$) greater in the conditioned media of keloid scar-seeded matrices, compared to that of normal scar-seeded matrices (Figure 4.12B, C). This was not found to be due to a significantly higher expression of the pro form of this enzyme by keloid scar cells (Figure 4.12A).

These results therefore, suggest that although MMP-2 clearly has an important role in collagen remodelling-induced apoptosis (seen from Figure 4.10, 4.11 and 4.12), it has no direct involvement in the apoptosis defect demonstrated by keloid scar cells. This might simply be explained by the possibility that MMP-2 activity, although essential for apoptosis, is not in itself sufficient to produce the required matrix breakdown products for the induction of apoptosis. It is likely that the activity of other MMPs is needed to coordinate the production of the necessary biochemical cues inducive to collagen contraction/remodelling-apoptosis. The activities of other MMPs were not studied, but could possibly show aberrant activation in keloid scar-derived fibroblasts. Alternatively, the products of matrix breakdown themselves may be insufficient to cause apoptosis of keloid scar cells, with keloid scar cells either requiring additional factors to allow them to undergo apoptosis or producing survival signals that inhibit apoptosis.

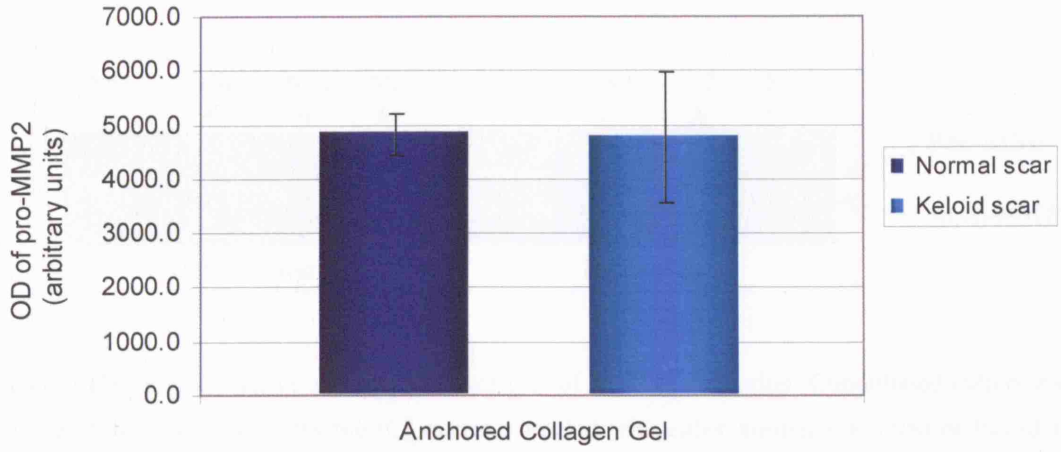


Figure 4.12A Densitometry of pro-MMP-2 expression detected by zymography. Conditioned culture media from anchored collagen matrices seeded with either normal scar or keloid scar-derived fibroblasts was assessed at day-7. 2-Way repeated measures ANOVA compared the expression of active MMP-2 in conditioned media from day-7 anchored vs contractile and keloid scar vs normal scar cell seeded gels.

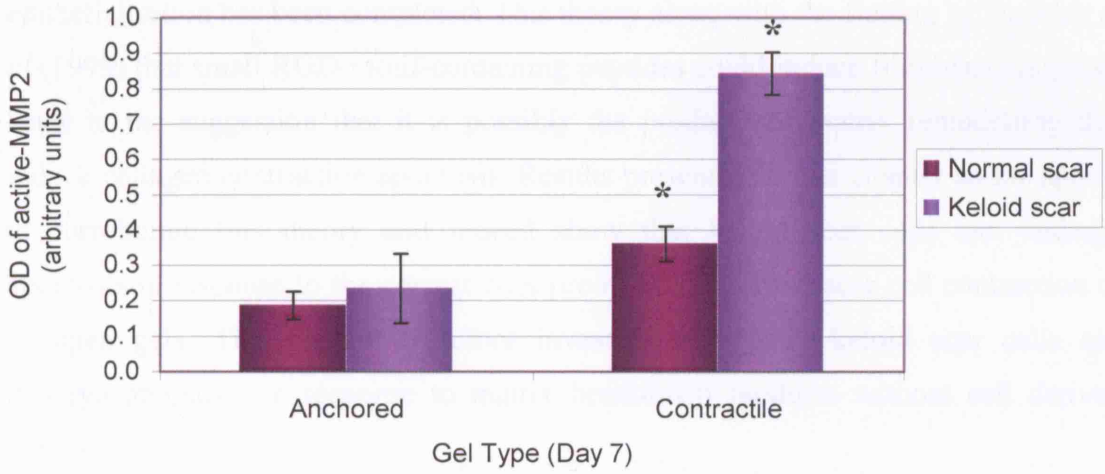


Figure 4.12B Densitometry of MMP-2 activation detected by zymography. Conditioned culture media from anchored (A) and contractile (C) matrices seeded with either normal scar or keloid scar-derived fibroblasts was assessed at day-7 of matrix culture for the expression levels of active MMP-2. Results represent the mean of n=6 each of normal scar and keloid scar samples. 2-Way repeated measures ANOVA compared the expression of active MMP-2 in conditioned media from day-7 anchored vs contractile and keloid scar vs normal scar cell seeded gels. *P=<0.001.

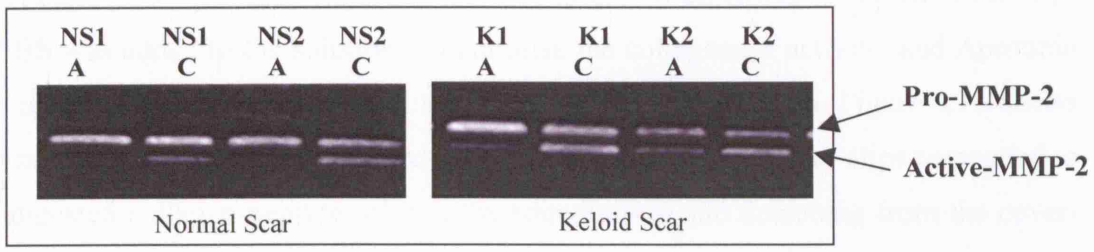


Figure 4.12C Representative zymographic analysis of MMP-2 activation. Conditioned culture media from anchored (A) and contractile (C) matrices seeded with either normal scar (NS) or keloid scar-derived (KLD) fibroblasts was assessed at day-7 of matrix culture for the expression levels of pro- and active MMP-2. Results represent 2x normal scar and 2x keloid scar cell strains.

4.2.3 Investigation of the Role of Matrix Breakdown Products in the Induction of Apoptosis

As previously discussed in section 3.1.1, Desmouliere *et al* (1995) proposed that factors selectively causing the death of fibroblastic cells could be liberated after epithelialisation has been completed. This theory along with the finding by Buckley *et al* (1999) that small RGD-motif-containing peptides could induce fibroblast apoptosis leads to the suggestion that it is possibly the products of matrix remodelling that induce collagen contraction apoptosis. Results presented in this chapter so far appear to corroborate this theory and indeed show that keloid scar cells can undergo apoptosis in response to the correct cues produced by normal scar cell contraction of collagen gels. This section therefore investigates whether keloid scar cells can undergo apoptosis in response to matrix breakdown products without cell derived factors.

4.2.3.1 Apoptosis in Response to Collagenase-D Cleaved Collagen Breakdown Products

To assess whether breakdown products from acellular collagen gels are capable of inducing apoptosis of normal scar- and keloid scar-derived fibroblasts, cell-free collagen matrices were digested with a collagenase (collagenase-D, derived from *Clostridium histolyticum*). After 30mins of digestion the collagen solution was then passed through a 10kDa molecular weight cut-off (MWCO) filter to provide a

collagen solution containing peptide fragments of <10kDa, theoretically containing small RGD-motif containing peptides. The collagenase treated collagen gel solution should not yield collagen fragments from cells grown on collagen-coated cover-slips as PBS was added to the solution to neutralise the collagenase activity, and Aprotinin was added to the enzyme solution to stop non-specific cell death. Figure 4.13 shows that normal scar fibroblasts cultured on collagen-coated glass cover-slips responded to the digested collagen-peptide solution by rounding-up and detaching from the cover-slip after 48hrs of treatment. In contrast, keloid scar-derived fibroblasts failed to respond to the collagen-peptide solution, the cells appeared healthy and well spread throughout the same time-course. Vital staining with propidium iodide confirmed that only normal scar-derived fibroblasts had undergone cell death after treatment with the collagen-peptide solution (Figure 4.13). The negative control solution (collagenase D solution alone) did not induce cell death in either scar type (Figure 4.13), suggesting that the cell death was specific to the digested collagen gel fragments.

4.2.3.2 Apoptosis in Response to Synthetic Small Soluble RGD-motif Containing Peptides

It is possible that RGD-motif-containing peptides are released during the proteolytic cleavage and remodelling of a collagen matrix. It is possibly these peptides that are conducive to apoptosis. To investigate whether keloid scar cells are resistant to the effect of synthetic RGD-peptides, these peptides or an inactive control peptide (RAD) were added to normal scar and keloid scar fibroblasts cultured on collagen-coated glass cover-slips. Figure 4.14 illustrates the effect of the synthetic RGD-peptide solution, where after 48hrs apoptosis is induced in the normal scar fibroblasts (green nuclei), but not in the keloid scar fibroblasts. The RAD peptide solution did not induce apoptosis in either scar type (Figure 4.5); only propidium iodide stained nuclei were present. These results demonstrate that keloid scar fibroblasts do indeed have an aberrant response to apoptosis inducing peptides, which are feasibly a product of ECM remodelling. These experiments were carried out to deduce whether RGD-peptides could induce apoptosis in normal scar and keloid scar cells and whether the same aberrant response to apoptosis cues was detected in keloid scar cells. Indeed, future experiments that quantify the apoptosis induced by RGD-peptides in keloid scar cells could be extrapolated and compared to that detected after collagen contraction-induced apoptosis of keloid scar cells.

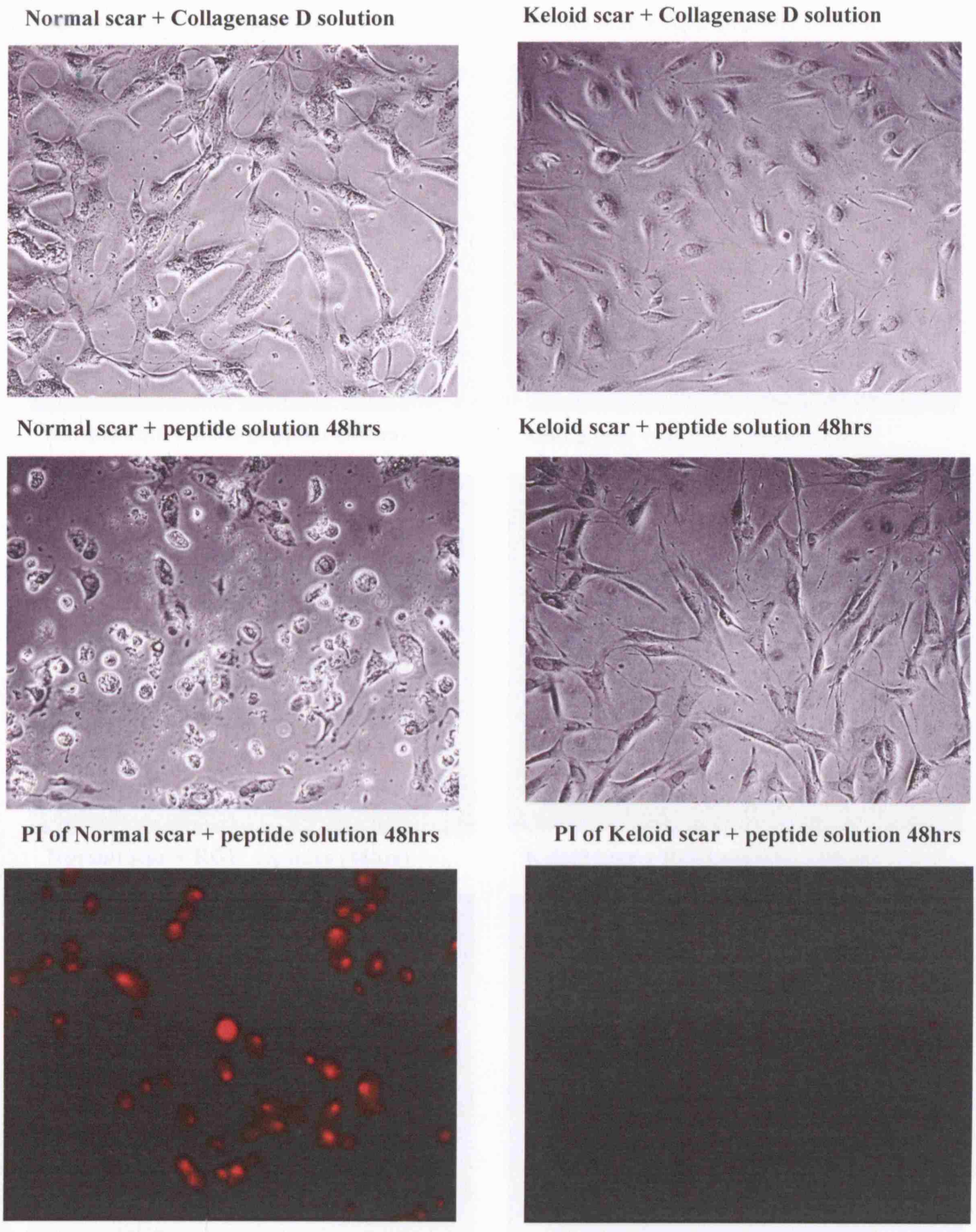


Figure 4.13 The effect of collagenase digested acellular collagen gels on monolayer cultured normal scar and keloid scar cells. Cell were cultured on collagen (10μg/ml) coated cover-slips in 6-well plates and maintained in SFM for 48hrs prior to adding either a collagenase D solution alone or digested (using collagenase D solution) acellular collagen gel for a further 48hrs. To prevent non-specific death both solutions was filtered (<10kDa MWCO) to remove large proteins, in addition aprotinin (100IU/ml) was added. Prodidium iodide (PI) vital staining labelled dead cells red. (x200 Mag).

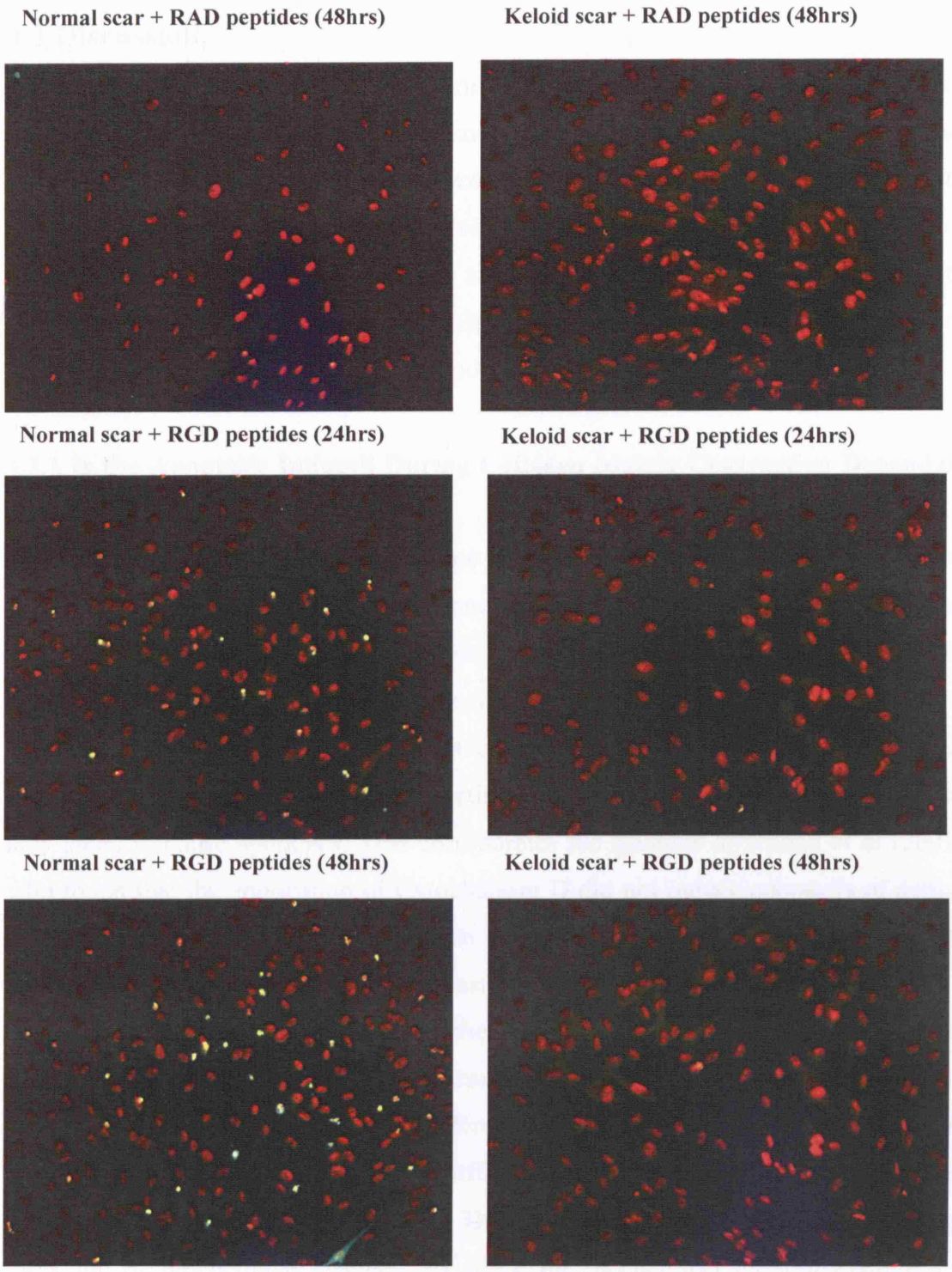


Figure 4.14 Typical ApoBrdU staining of normal scar and keloid scar fibroblasts after the treatment with RGD or RAD (control) peptides. Cells were cultured in SFM containing 1mM of the inactive control peptide (RAD) or 1mM of RGD peptide. Cells were cultured on collagen (10μg/ml) coated glass cover-slips in 6-well plates in SFM for 48hrs prior to adding relevant peptide solutions. After 24hrs and 48hrs of peptide treatment cells were analysed by TdT end-labelling for apoptotic nuclei (green) and red (propidium iodide) counterstaining. This experiment was carried out in triplicate with n= 6 normal scar and keloid scar cell strains. (x200 Mag).

4.3 Discussion

Although induction of apoptosis during fibrillar collagen contraction is well established, its mechanisms remain unknown. Results from Chapter 3 of this thesis demonstrate that although matrix contraction does occur with keloid scar fibroblasts, apoptosis does not. This suggests that loss of cell tension and the act of contraction itself is insufficient to trigger apoptosis, at least in the case of keloid scar fibroblasts. The work presented in this chapter examined the contribution of both mechanical and biochemical events in the induction of apoptosis.

4.3.1 Is the Apoptosis Induced During Collagen Matrix Contraction Dependant on Mechanical Cues Alone?

The results from this chapter demonstrate that the dissipation of cell tension and loss of cell-cell and cell-matrix contacts alone are not sufficient to induce normal scar-derived fibroblasts to undergo apoptosis. In particular, attempts to induce classical anoikis over the same time-course usually taken for collagen contraction-induced apoptosis failed to instigate cell death of cells derived from either scar type. In addition, cytochalasin D disruption of actin cytoskeleton of cells within collagen gels also failed to cause apoptosis. This corroborates the findings of Niland *et al* (2001), who found that the application of Cytochalasin D did not induce apoptosis of dermal fibroblasts seeded in anchored collagen gels and actually inhibited the apoptosis induced by gel contraction. In contrast, Grinnell *et al* (1999) reported that a combination of serum deprivation together with cytochalasin D treatment of dermal fibroblasts seeded in anchored gels caused a small but significant increase in apoptosis (12-15%). Despite these different results, it is still clear that whilst the dissipation of cell tension is in itself insufficient, it still has a role in the apoptosis that is induced during collagen contraction. This role is probably an indirect one through induction of biochemical changes. Evidence for biochemical cues of apoptosis is provided by the experiment in section 4.2.1.2, where a homogenised preparation of a collagen gel that had been contracted by normal scar cells, induced apoptosis of monolayer cultures of normal scar cells in the absence of any mechanical stimulus.

4.3.2 Are Keloid Scar Fibroblasts Able to Respond to or Produce the Biochemical Cues of Collagen Contraction?

To examine if keloid scar cells could respond to the correct biochemical cues of collagen contraction-induced apoptosis, keloid scar cell monolayers were tested to see if they responded to the homogenised collagen gel preparations that had been contracted by normal scar cells. Keloid scar cells did indeed undergo apoptosis and to an equivalent level to that seen in normal scar cell monolayers. The reverse experiment was also performed to examine if keloid scar cells were able to produce the correct biochemical cues for apoptosis. Specifically, homogenised preparations of collagen gels that had been contracted by keloid scar-derived fibroblasts were tested for their ability to induce apoptosis of monolayer cultures of normal scar cells. No evidence of apoptosis was seen. These results clearly indicate that keloid scar cells are capable of undergoing apoptosis in response to the correct cues; however they fail to produce the necessary conditions for apoptosis induction. The question that remains however, is what these biochemical cues are?

4.3.3 Potential Clues to the Biochemical Apoptosis Signals

One of the major biochemical differences between anchored and contractile collagen gels is with regard to collagenase activity. A potential role for the action of collagenases and other matrix-degrading enzymes has been indicated by the work of Buckley *et al* (1999), where potential ECM breakdown products (small soluble RGD-motif-containing peptides) have been shown to directly induce apoptosis of fibroblasts. This led to the hypothesis that the apoptosis defect exhibited by keloid scar cells was simply due to their failure to degrade the matrix and produce RGD-containing peptides that induce apoptosis. The degradation of collagen gel proteins was therefore examined crudely by comparing the banding patterns of protein lysate preparations via SDS-PAGE.

Silver staining of day-7 contractile and anchored collagen gel preparations demonstrated an increase in small molecular weight polypeptide species (<50kDa) and a concomitant decrease in higher molecular weight polypeptides (50 – 150kDa) in collagen gels that had been contracted by normal scar cells compared to those in either anchored gels or those contracted by keloid scar cells. Indeed, the protein profile of the contractile collagen matrices conditioned by keloid scar fibroblasts was

identical to that of the anchored collagen matrices conditioned by either cell type. This result suggests that in agreement with published observations of an increased collagenase activity (Lambert *et al.*, 1992, 2001; Langholz *et al.*, 1995; Ravanti *et al.*, 1999b) in contractile collagen gels, there appears to be an increase in the degradation of the matrix during contraction of normal scar cell-seeded gels. This apparent degradation is not seen in gels that have been contracted by keloid scar cells.

4.3.4 Are MMPs and TIMPs Involved in the Apoptosis that Takes Place During Collagen Matrix Contraction?

Due to the possibility of the involvement of small breakdown products of matrix remodelling in the initiation of apoptosis during collagen contraction, it was further hypothesised that MMP action might also play a role. Inadequate MMP activity by keloid scar cells may explain their failure to produce apoptotic cues.

Blocking MMP activity using a broad-spectrum MMP inhibitor (Ilomastat, blocking MMP-1, 2, 3, 8 and 9), and a specific MMP-2 inhibitor was shown to completely abrogate the apoptosis induced during collagen contraction, whilst having no obvious effect on actual contraction. However, Ilomastat is known to also inhibit the activity of ADAMs and ADAMTSs (Kang *et al.*, 2004), and the effect of the specific inhibitor of MMP-2 on these enzymes is, to the author's knowledge, unknown. For this reason a physiological inhibitor of MMPs (and particularly of MMP-2), recombinant human TIMP-2, which is known not to inhibit ADAMs and ADAMTSs was used to assess its effect on collagen contraction-induced apoptosis. This too completely inhibited any apoptosis. Nevertheless, these results are perhaps complicated by a report that suggests that TIMP-2 may exhibit anti-apoptotic properties on monolayer cell cultures. Valente *et al* (1998) describes that over-expressing TIMP-2 transfected melanoma cells were more resistant to apoptosis induction by the cytotoxic drug mitomycin. However, this method of apoptosis induction may have no relevance to the healing wound. In contrast, other authors report that TIMP-2, unlike TIMP-1, has no effect on apoptosis of both breast epithelial and B cells (Guedez *et al.*, 1998; Liu *et al.*, 2003). The methods of induction of apoptosis in these reports were various and included, anoikis, serum deprivation, staurosporine, cold-shock, anti-FAS, and γ-irradiation. Again the relevance of these results to the apoptosis of fibroblasts in a model of wound healing is unclear. Nonetheless, the findings presented in this chapter

appear to concur that MMP activity and specifically MMP-2 activity is required for the apoptosis signals induced during collagen contraction. Furthermore, these results suggest that the role of MMPs in apoptosis is not simply through an effect on contraction.

The effect of MMP inhibition on gel contraction has been examined previously by other groups; all three report a reduction in contraction (Daniels *et al.*, 2003; Mirastschijski *et al.*, 2004; Wong *et al.*, 2004). However, Mirastschijski *et al* (2004) assessed the effects of Ilomastat on excisional wounds in rats, contraction of which is brought about by both keratinocytes and fibroblasts, and Wong *et al* (2004) studied Ilomastat's effect on lens capsule contraction, which is carried out by epithelial cells. Whilst the Daniels *et al* (2003) publication, using Tenon's capsule fibroblasts also reported MMP-inhibition affecting collagen-matrix contraction, this was done using much higher concentrations of Ilomastat (100μM); which whilst non-toxic to cells cultured in monolayer, this level of Ilomastat produces death of cells in collagen gels (unpublished observation – C. Linge). Daniels *et al* (2004) did not examine the viability of the cells and thus their observation could be explained by a toxic effect leading to a reduction in cell numbers. The results presented in this chapter used MMP inhibitors at concentrations, which although still effective at inhibiting MMPs, were not toxic to cells seeded in matrices.

Further analysis by RT-PCR of the four main MMPs (MMP-1, -2, -3 and -13) known to be produced by fibroblasts and involved in tissue remodelling and repair, appeared to reveal no significant differences between normal scar and keloid scar fibroblasts after collagen matrix culture. It was clear to see that a significant ($p<0.001$) increase in gene expression of all MMPs examined occurred in contractile collagen matrices, compared to anchored and T0 collagen matrices. This result is in agreement with Lambert *et al* (1992, 2001) who found that MMP-1 and MMP-2 were upregulated in collagen gels that had been contracted by human skin fibroblasts.

On analysis of the gene expression levels of the TIMPs, only TIMP-1 and 2 appeared significantly increased in contractile gels compared to T0 for both keloid scar and normal scar cells. However, there was no significant difference in TIMP expression levels in contractile gels between the two scar types. Interestingly, TIMP-2 is also

significantly elevated in anchored gels containing normal scar cells, which might explain the reduced collagenase activity seen in these gels. However, this is not seen in anchored gels containing keloid cells. TIMP-3 and 4 appeared not to be affected by collagen matrix culture in either anchored or contractile matrices seeded with normal scar cells. Keloid scar fibroblasts similarly showed no significant change in TIMP-4 expression after collagen matrix culture; however, TIMP-3 was significantly increased in contractile gels. Irrespective of this apparent significant difference, the TIMP-3 results were deemed unreliable, since there was significant variation between cell-strains of the same type, and thus warrants no further discussion. This is possibly because TIMP-3 was expressed at extremely low levels; the small differences detected between the different cell-strains therefore, may have led to significant differences.

These results show no obvious differences between keloid and normal scar cells in contractile gels that might explain the failure of keloid scar cells to remodel collagen and perhaps to produce potential cues of apoptosis. Indeed, more specific analysis of mRNA gene expression levels needs to be carried out before conclusive evidence of this, possibly using quantitative RT-PCR. However, gene expression is not necessarily paralleled by translation into protein or indeed the activation of these enzymes. Investigations were therefore continued to assess the protein level and activity of MMPs during collagen-contraction by both normal scar and keloid scar fibroblasts. MMP-2 was chosen for further investigation, since its specific inhibition was shown to block apoptosis and its action is most likely to produce the types of small soluble polypeptides which could feasibly be involved in collagen contraction-induced apoptosis.

Investigations to assess the activity of MMP-2 demonstrated that MMP-2 was significantly activated in contractile collagen gels versus anchored collagen gels, regardless of scar type. Surprisingly, there was no significant difference in the level of pro-MMP-2 protein expressed between normal scar and keloid scar fibroblasts but, the levels of active MMP-2 were actually found to be significantly increased (2.5-fold) in keloid scar cells.

This result indicates that as well as having relatively normal MMP and TIMP gene expression, the protein secretion and activation of MMP-2 at least, is not reduced in

keloid scar-derived fibroblasts. Ideally, further investigation of other ECM-degrading proteases needs to be carried out before concluding that keloid scar fibroblasts do not display any fault in their enzymatic ability to degrade extracellular matrices. Nonetheless, the results of the studies presented here suggest that faulty gene expression of the MMPs studied or activity of MMP-2 may not explain the apoptosis defect identified in keloid scar cells.

4.3.5 Response of Scar Cells to Matrix Breakdown Products Potentially Involved in Apoptosis

Investigations were carried out to determine the action of breakdown products from ECM remodelling which could potentially lead to the induction of apoptosis. Both collagen breakdown products (from collagenase digested acellular collagen gels) and pure synthetic RGD-containing peptides (GRGDNP) were used to assess if apoptosis could be induced in scar-derived fibroblasts. The effects of these breakdown products were examined on monolayer cultures of normal scar and keloid scar fibroblasts seeded on collagen-coated cover-slips in tissue culture plates, in a similar fashion to the original RGD peptide studies performed by Buckley *et al* (1999). The collagen coating was used in an attempt to reproduce the same integrin expression and signalling as that in collagen matrix culture and also, to ensure that cell attachment was RGD-independent (Hayman et al 1985). Although RGD-motifs are found on collagen type I, due to the 3-D arrangement of the collagen fibrils these are hidden from cells. Cells bind more readily to RGD-motifs expressed by fibronectin, as these are clearly displayed on fibronectin molecules. The effect of both the collagen breakdown products and the RGD-peptides on monolayer cultured fibroblasts were identical, being comparable with the results of collagen contraction induced apoptosis (Chapter 3), whereby normal scar fibroblasts underwent apoptosis but keloid scar fibroblasts failed to respond.

This failure of keloid scar cells to respond to collagenase-digested collagen and RGD peptides, suggests that keloid scar-derived fibroblasts may be unable to respond to the biochemical apoptosis cues of collagen contraction. Although it remains to be proven that these cues comprise of matrix degradation products. These results are in contrast with the earlier findings that keloid scar cells are able to respond to the apoptosis signals produced during normal scar cell-contraction of collagen gels (Figure 4.5).

This may indicate that normal scar cells remodel and condition a contractile-collagen gel producing not only ECM cleavage products but also other cell-derived factors which may affect apoptosis; factors which keloid scar cells may fail to express. Clearly, the collagen matrices contain traces of a large variety of other proteins such as fibronectin, and proteins secreted by the cells such as glycosaminoglycans and growth factors, all of which possess the capability of affecting apoptosis. Alternatively, normal scar cells are able to cleave specific sized peptides that are not cleaved by collagenase digestion of acellular collagen gels or by keloid scar cell-conditioned collagen gels. Further experiments characterising the size of peptide fragments produced by normal scar- versus keloid scar-cell conditioned contractile collagen gels, and versus collagenase-digested collagen fragments would help to determine this.

4.3.6 Summary

In summary, results from this chapter have determined two possible reasons as to why keloid scar-derived fibroblasts fail to undergo collagen contraction-induced apoptosis: 1) keloid scar-derived fibroblasts do not appear to be sensitive to synthetic RGD-motif containing peptides or collagenase digested collagen fragments without the presence of an additional element that is produced by normal scar cells during contraction of collagen, 2) keloid scar fibroblasts do not appear to remodel the collagenous matrix to the same extent as normal scar-derived fibroblasts, with inadequate cleavage and production of small polypeptides.

The next chapter investigates the role myofibroblast differentiation plays in the ability of cells to undergo apoptosis in response to collagen-contraction.

Section B

Chapter 5

Is Myofibroblast Differentiation Required for Successful Collagen Contraction-Induced Apoptosis?

5.1 Introduction

The results presented so far in this thesis demonstrate that when fibroblasts derived from normal scar tissue are allowed to contract a 3-dimensional collagen gel, a significant proportion of them (30-60%) undergo apoptosis. However these cells do not respond in the same way when contracting fibrin gels. In addition, keloid scar-derived cells, although capable of obvious collagen gel contraction do not undergo detectable apoptosis. It has previously been hypothesised that it is specifically the myofibroblast phenotype that undergoes apoptosis to the cues of granulation tissue remodelling (Darby *et al.*, 1990; Clark, 1993b; Desmouliere *et al.*, 1995). Results from electron microscopy, immunohistochemistry and *in situ* end labelling of cells within wounds revealed that it is specifically cells expressing α-SMA (myofibroblasts) that begin to show typical features of apoptosis; and at around the 20th to 25th day after wounding there is a reduction in α-SMA in wound tissue (Darby *et al.*, 1990; Desmouliere *et al.*, 1995). If this is true, this may explain why certain cells under specific conditions are sensitive to apoptosis cues whereas other cells are not. Furthermore, it is possible that the different level of apoptosis that occurs under different conditions is simply a function of the level of myofibroblast differentiation that has taken place.

This chapter therefore concentrates on investigating whether the degree of myofibroblast differentiation determines the level of apoptosis that occurs in response to collagen-contraction.

In order to understand the potential reasons why the myofibroblast phenotype might be preferentially targeted by apoptosis cues, a review of the literature with regards to what is known of their origins, functions and matrix interactions needs to be carried out.

5.1.1 About the Myofibroblast

5.1.1.1 The Force Generating Cell? – The Myofibroblast

Research carried out in the 1960s implicated connective tissue cells in the generation of the tensile strength responsible for wound contraction (Abercrombie *et al.*, 1960). Other studies found that force generating cells were localised at the wound margins rather than in the centre of the granulation tissue (Watts *et al.*, 1963). Nevertheless, it was Gabbiani *et al.* (1971) who first discovered the myofibroblast cell type by ultramicroscopy. Further, in

1972 Gabbiani *et al.* found that strips of granulation tissue were able to undergo smooth muscle-like contraction *in vitro* and found that the cells within the granulation tissue exhibited features of smooth muscle cells, such as actin filament bundles (stress fibres). These 'myo-fibroblasts' were proposed to be responsible for the force generation; their presence is now acknowledged to be a general feature of tissue undergoing contraction (Skalli *et al.*, 1987; Rudolph *et al.*, 1991).

5.1.1.2 The Myofibroblast and Focal Adhesion Complexes

Myofibroblasts express numerous fibronexus junctions, which suggest myofibroblasts form very tight adhesions to the surrounding fibronectin-rich granulation tissue (Singer *et al.*, 1984; Tomasek and Haaksma, 1991). It has been suggested by Darby *et al.* (1990) and Welch *et al.* (1990) that the timing of the switch from a fibroblast phenotype to that of a myofibroblast depends in part on the wounds resistance to contraction. It is postulated that the migrating fibroblasts at the wound margins generate sufficient force to initiate wound contraction. As contraction continues, resistance increases, migrating fibroblasts begin to differentiate into myofibroblasts, with both the actin cytoskeleton and the cells slowly becoming organised along the lines of least resistance, minimising cell-stress (Figure 5.1) (Darby *et al.*, 1990; Welch *et al.*, 1990).

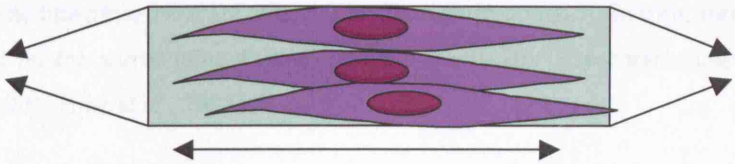


Figure 5.1 Alignment of cells along lines of least resistance, minimising the stress on the cell.

The contractile machinery of myofibroblasts is organised as bundles of microfilaments which appear similar to stress fibres present in cultured fibroblasts. These actin bundles terminate at the myofibroblast surface in the fibronexus, they are also known as mature or supermature focal adhesion complexes (FACs) (Dugina *et al.*, 2001). FACs are specialised adhesion complexes that utilise transmembrane integrins to link intracellular actin with extracellular fibronectin domains, as previously discussed in section 4.1 and illustrated in Figure 5.2.

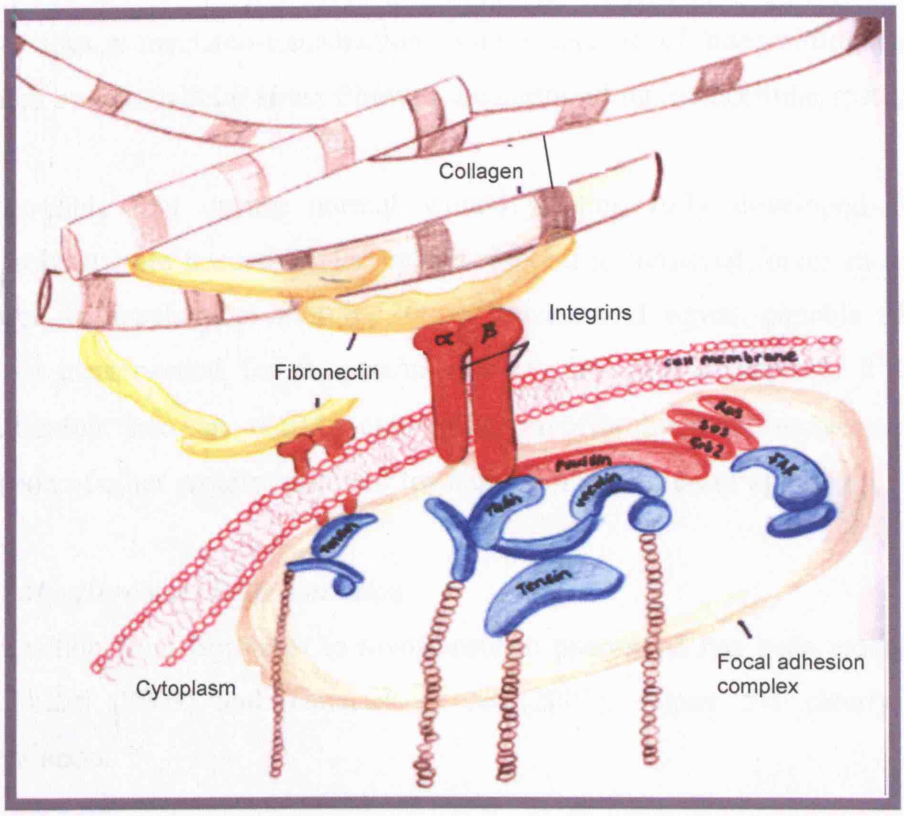


Figure 5.2 Illustration of how a force applied upon a cell is translated from the inside of the cell to the outside. Stress is converted within the cell via an intracellular focal adhesion complex (FAC), which is linked to transmembrane integrins, these are attached to fibronectin domains. In turn, force is carried out of the cell and is inflicted on the surrounding collagen protein fibrils, thereby contracting the matrix (adapted from Dugina *et al.*, 2001; Hinz *et al.*, 2003).

The major components of mature FACs include: vinculin, paxillin, talin, α-actinins, focal adhesion kinase (FAK), cytoplasmic β- and γ-actins and αvβ3 integrin (Geiger *et al.*, 2001). Supermature focal adhesions have also been found to contain considerable amounts of tensin, α-SMA, α5β1 integrin and extracellular ED-A Fibronectin (Dugina *et al.*, 2001; Hinz *et al.*, 2003).

It has been found by a number of research groups that cultured fibroblasts also develop FACs that organise fibronectin fibrils and which, unlike mature FAs but like supermature FAs, contain tensin and α5β1 integrin; however they do not contain vinculin (Geiger *et al.*, 2001). Consequently, supermature FAs are reliably identified *in vitro* by the presence of intracellular α-SMA stress fibres associated with extracellular ED-A fibronectin and by the

increased presence of both tensin and vinculin (Hinz and Gabbiani, 2003). Functionally, this provides a mechano-transduction system capable of transmitting the force that is generated by intracellular stress fibres to the surrounding extracellular matrix.

It is possible that during normal wound healing fully developed FACs (seen in myofibroblasts) are needed for the cell to respond to tensional forces in such a way as to transduce the mechanical stimulus into a biochemical signal, capable of producing the apoptosis cues needed for successful cell clearance. Alternatively, it is possible that differentiation into an α-SMA-expressing myofibroblast is accompanied by altered expression of other proteins required for induction/inhibition of apoptosis.

5.1.1.3 Myofibroblast Differentiation

The transition from fibroblast to myofibroblast phenotype has been extensively reviewed by Gabbiani (2003) and Tomasek *et al.* (2002), Figure 5.3 clearly illustrates this differentiation.

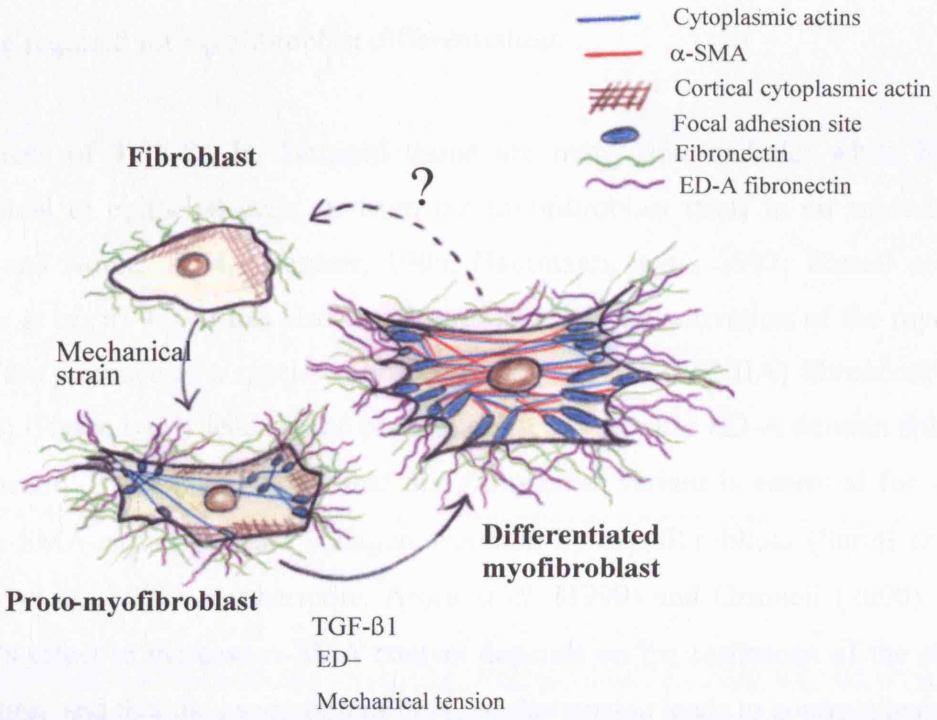


Figure 5.3 The two stage model of myofibroblast differentiation, showing fibroblast differentiation to proto-myofibroblast then to fully differentiated myofibroblast (adapted from Tomasek *et al.*, 2002).

Under mechanical stress, fibroblasts differentiate into proto-myofibroblasts, which form cytoplasmic actin-containing stress fibres that terminate in fibronexus adhesion complexes. Proto-myofibroblasts are also capable of expressing and organising cellular fibronectin as well as generating force. TGF-β1 can increase the expression of ED-A fibronectin. Both factors in the presence of mechanical stress initiates the full differentiation of a myofibroblast. This is characterised by extensively developed α-SMA stress fibres and by the development of large fibronexus adhesion complexes (*in vivo)* or supermature focal adhesions (*in vitro*) (Tomasek *et al.*, 2002).

The *in vivo* activation of myofibroblasts, as signified by the development of α-SMA containing stress-fibres, may be through a separate signalling mechanism to that of cell proliferation. TGF-β1 is known to support the differentiation of fibroblasts into the myofibroblast phenotype (Hinz and Gabbiani, 2003). TGF-β1 is also known to signal fibroblast proliferation during the early phases of wound healing. It is possible that early on in wound healing TGF-β1 acts to mediate the proliferation of migratory cells, once these cells have reached the wound site and have become stationary; TGF-β1 then mediates the signalling required for myofibroblast differentiation.

The sources of TGF-β1 in damaged tissue are many and include: white blood cells, parenchymal or epithelial cells, or from the myofibroblast itself in an autocrine fashion (Border and Noble, 1994; Gressner, 1996; Hautmann *et al.*, 1997; Bissell *et al.*, 1995; Clouthier *et al.*, 1997). It has also been suggested that the activation of the myofibroblast requires the presence of a specific fibronectin variant, ED-A (EIIIA) fibronectin (Serini *et al.*, 1998). Tissue injury leads to the production to this specific ED-A domain splice variant of fibronectin. It has been shown that this fibronectin variant is essential for TGF-β1 to trigger α-SMA expression and collagen secretion by myofibroblasts (Serini *et al.*, 1998; Tomasek *et al.*, 2002). Furthermore, Arora *et al.* (1999) and Grinnell (2000) found that TGF-β1's effect to increase α-SMA content depends on the resistance of the substrate to deformation, and that the generation of intracellular tension leads to contractile cytoskeletal gene expression.

Following myofibroblast activation, PDGF or connective tissue growth factor (CTGF) (Bradham *et al.*, 1991), have been reported to be the primary factors responsible for

myofibroblast proliferation (Grotendorst, 1997; Jobson *et al.*, 1998; Mallat *et al.*, 1998). TGF-β1 was once considered the principal factor, however it is now thought that TGF-β1 acts predominantly through the induction of PDGF receptors or synthesis of CTGF by myofibroblasts (Gressner, 1996; Sappino *et al.*, 1990; Schmitt-Graff *et al.*, 1994). In light of these facts, TGF-β1 and ED-A fibronectin could be preferential targets for apoptosis cues.

The Ras superfamily member Rho, (a small guanosine triphosphatase (GTPase)) has been implicated in the regulation of the formation of fibronexus and stress fibre assembly, specifically in mammalian cells by RhoA (Hall, 1998). The small monomeric GTP-binding protein Rho, also regulates myofibroblast morphology (Yee, 1998; Powell *et al.*, 1999). Thus, the Ras superfamily may also be implicated in the apoptosis of wound cells.

Fully activated myofibroblasts have further been proposed to exist in a distinct morphological state. It has been suggested that the myofibroblast is able to either de-differentiate back into a quiescent fibroblast phenotype or (if the myofibroblast is an irreversible phenotype) further transform into a distinct morphological state, the 'stellate-myofibroblast' (Powell *et al.*, 1999) (Figure 5.4). This dendritic-like cell-type is detected in mechanically relaxed 3-D collagen matrices (Hinz *et al.*, 2004), whether these cells also express α-SMA is not clear from the literature. The 'fully activated' myofibroblast appears enlarged with thickened cell-extensions in a bipolar orientation in comparison to the 'stellate myofibroblast', which appears stellate with numerous spindle-like extensions (Powell *et al.*, 1999).

TGF-β, PDGF, IGF-II and IL-4 appear to be important growth factors involved in the differentiation of fibroblasts to myofibroblasts or of stellate myofibroblasts into activated myofibroblasts (Lund *et al.*, 1996; Tang *et al.*, 1996; Clouthier *et al.*, 1997; Doucet *et al.*, 1998). Conversely, IFN-α and IFN-γ decrease the expression of α-SMA in myofibroblasts (Desmouliere *et al.*, 1992; Guido *et al.*, 1996). However, it is not clear whether myofibroblasts are able to re-differentiate back into quiescent fibroblasts, or whether they irreversibly undergo further transformation, in this manner becoming a terminally differentiated cell type, potentially responsive to apoptosis cues.

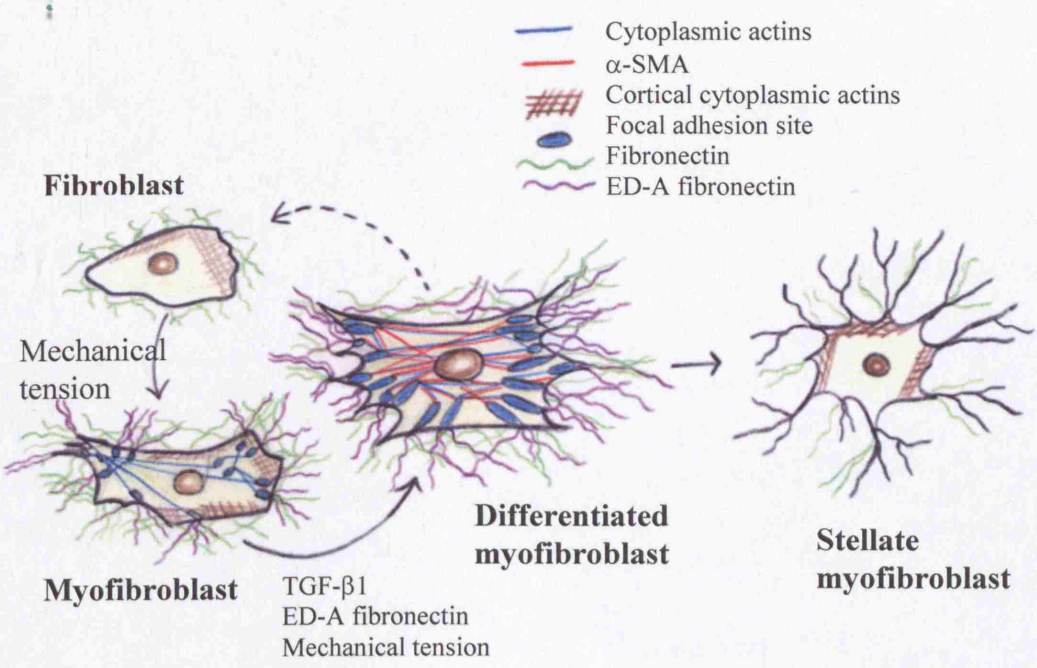


Figure 5.4 Proposed scheme for the transition of a fully activated myofibroblast to a stellate myofibroblast (adapted from Powell *et al.*, 1999 and Tomasek *et al.*, 2002).

5.1.2. The Role of the Myofibroblast during Wound Healing

The myofibroblast would appear to have multiple roles during wound healing, some of which may be essential to the success of wound healing, as they are ubiquitous in the healing of most tissue types.

5.1.2.1 Contraction

Healing is thought to be facilitated by the fact that the myofibroblasts are contractile, these cells are considered to act to reduce the amount of denuded surface area of wounded tissue. The ability of myofibroblasts to accumulate and extrude calcium, allows them to do this. This activity causes cyclic increases and decreases in calcium concentrations, allowing actin-myosin contraction (Furuya *et al.*, 1994). The electrical signals created by cyclic ion movements can be transmitted through the gap junctions on neighbouring cells. In this manner, the cytoplasmic continuity of the cells allows the electrical signals to be transduced throughout the tissue (Powell *et al.*, 1999).

5.1.2.2 Role in the Inflammatory Process

Myofibroblasts have a very influential role on the inflammatory response. Myofibroblasts affect the inflammatory response through their ability to produce abundant amounts of cytokines and chemokines (Clark, 1996). Activated myofibroblasts also express immunoglobulin-like cell adhesion molecules such as: ICAM-1, VCAM and neural cell adhesion molecule (NCAM) (Clark, 1996). Thus, inflammatory cells like lymphocytes, mast cells and neutrophils are able to attach to myofibroblasts and contribute in the immunological and inflammatory response (Crowston *et al.*, 1997; Fiocchi, 1997; Hogaboam *et al.*, 1998).

The expression of α and β integrins, also allows myofibroblasts to bind to extracellular matrix proteins. Through these properties myofibroblasts participate in the formation of granulomas (Desmouliere and Gabbiani, 1996). Granulomas are the description given to cells which produce copious amounts of cytokines and other inflammatory mediators (Isaji *et al.*, 1994). Persistence of the myofibroblast phenotype may therefore lead to an overactive wound-site and a failure of the repair process to resolve.

5.1.2.3 Production of Extracellular Matrix Proteins and other Components

During wound healing, myofibroblasts function to contribute to the structure of the wound as well as the growth and differentiation of wound cells, through the production of matrix molecules such as collagen, glycosaminoglycans, tenascin, fibronectin and matrix modifying proteins (Desmouliere and Gabbiani, 1996). When unchecked these processes may lead to pathological tissue fibrosis, which is why it is important that these cells are de-activated once their role is complete and are signalled to undergo apoptosis.

5.1.3 The Origin of the Myofibroblast

On studying the origin of these myofibroblasts, it was suggested from analysis of cytoskeletal markers that they are derived from fibroblasts rather than smooth muscle cells (Eddy *et al.*, 1988; Darby *et al.*, 1990). Interestingly however, there have been suggestions that at least a sub-population of the α-SMA expressing cells present within the wound site may possibly originate from pericytes or even progenitor stem cells in circulation (Sappino *et al.*, 1990; Forbes *et al.*, 2004). In an investigation where a female patient received a bone marrow transplant from a male donor and subsequently developed hepatitis-C induced

cirrhosis, 12.4% of the α-SMA expressing cells found in the liver were determined to be Y chromosome positive, indicating a bone-marrow origin (Forbes *et al.*, 2004). Although this study examined liver fibrosis, not dermal fibrosis, it nevertheless highlights the potential involvement of extra-parenchymal cells in tissue repair. Even so, during granulation tissue remodelling, migrating fibroblasts have been found to differentiate into myofibroblasts (Darby *et al.*, 1990; Welch *et al.*, 1990).

5.1.4 Investigating the Role of the Myofibroblast in the *In Vitro* Wound Healing Model

When fibroblasts are cultured within 3-D collagen matrices the cells are maintained within an anchored matrix for 4-days prior to matrix release. During this early period they become stressed, express α-SMA-containing stress fibres (Arora *et al.*, 1999; Bride *et al.*, 2004), and a mainly bipolar morphology (as described previously in chapter 3); in essence they become classical myofibroblasts. If, as proposed by others (Darby *et al.*, 1990; Clark 1993b; Desmouliere *et al.*, 1993), it is these cells alone that undergo apoptosis, then the extent of apoptosis may be regulated by the level of myofibroblast differentiation.

Theoretically, keloid scar-derived fibroblasts may have an inherent fault in their ability to differentiate into myofibroblasts under the cues of stressed-relaxed collagen matrix culture. Thus, if myofibroblasts are required to respond to apoptosis cues then keloid scar cells may be unable to respond to the apoptosis induction cues of collagen-contraction. Alternatively, it is possible that failure of keloid scar fibroblasts to differentiate into myofibroblasts may lead to an inability to produce collagen contraction apoptosis cues.

It is known from previous research that the addition of TGF-β1 to the culture medium of fibroblasts signals the cells to express vast amounts of α-SMA containing stress fibres, thus signalling the cells to differentiate into myofibroblasts (Vaughan *et al.*, 2000; Hinz and Gabbiani, 2003; Thannickal *et al.*, 2003). This also occurs for cells derived from normal and pathological scars in monolayer culture (Dr C Linge, personal communication).

It is hypothesised therefore, that the addition of exogenous TGF-β1 to the *in vitro* wound healing model will significantly increase the apoptosis that occurs during collagen gel contraction due to increased myofibroblast differentiation.

5.1.5 Aim

The work presented in this chapter investigates the role of the myofibroblast phenotype in collagen contraction-induced apoptosis.

5.2 Results

5.2.1 Does the Sensitivity to Apoptosis Depend on the Presence of the Myofibroblast Phenotype?

To determine whether the presence of the myofibroblast phenotype correlates with apoptosis induction specific to matrix contraction, *in situ* staining for α-SMA was carried out on day-7 anchored and contractile matrices of both collagen gels and fibrin gels as a control. It is important to note that obtaining clear micrographs of the FITC fluorescent microscopy staining was difficult due to the 3-D nature of the gels, and the density of the cells. Nevertheless, stress fibres were clearly evident under different conditions.

Normal Scar Cells

Figure 5.5 shows that when normal scar fibroblasts are seeded within anchored collagen matrices, numerous prominent, enlarged α-SMA-positive myofibroblasts are present. Many of the cells appeared bipolar in orientation and also seemed to have enlarged positively stained cell bodies. The α-SMA stained cells were evenly dispersed throughout the matrix (Figure 5.5). Within the contractile collagen matrices at day-7 the pattern of α-SMA staining had changed dramatically. The positively-stained cells appeared very fragmented and stellate, with long spindle-like cell processes (see arrows Figure 5.5). There was also very little cytoplasm around the nucleus compared with cells in anchored gels. It is important to note that the cells seeded into the collagen gels were initially fibroblasts and not myofibroblasts.

Figure 5.6 illustrates immunohistochemical staining for α-SMA with normal scar-derived fibroblasts cultured within a fibrin matrix. The normal scar fibroblasts seeded with anchored fibrin matrices did appear to express α-SMA (Figure 5.6). The cells appeared very similar to those in anchored collagen matrices; they were bipolar in orientation and very taut. Although the cells that stained positive for α-SMA appeared equally dispersed throughout the anchored fibrin matrix (Figure 5.6), the staining was slightly weaker than that of normal scar cells in anchored collagen matrices (Figure 5.5). Within the contractile

fibrin matrices slightly fewer cells stained positive for α-SMA. The cells that did stain positive for α-SMA appeared shrunken with less cytoplasm around the nucleus compared to cells within anchored fibrin matrices (Figure 5.6). The normal scar cells appeared relatively healthy within the contractile fibrin matrices in comparison to those in the contractile collagen matrices; with no obvious fragmentation of the cells (Figure 5.6). Nonetheless, the cells did not appear to be held as taut within the matrix as cells within anchored fibrin matrices, judged by the relaxed appearance of cell processes. The normal scar cells appeared stellate and closely linked together by their cell extensions within contractile fibrin matrices (Figure 5.6).

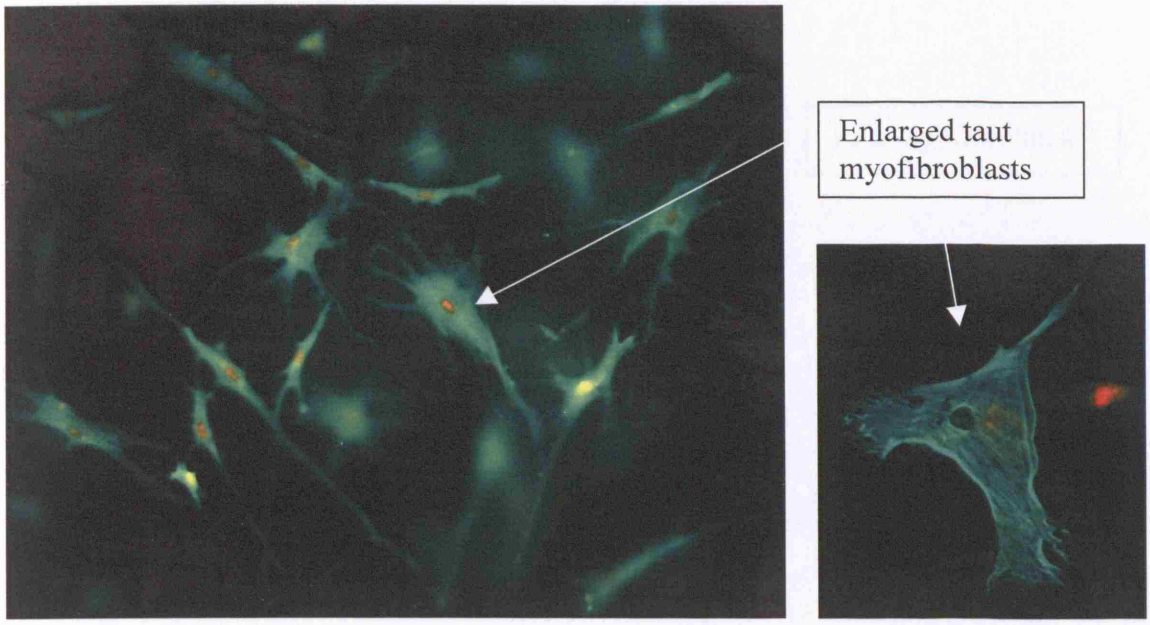
To confirm this staining, confocal microscopy was carried out. Figure 5.7 illustrates normal scar fibroblasts within collagen and fibrin matrices, of both anchored and contractile matrix types. There is positive α-SMA staining of fibroblasts seeded within both collagen and fibrin matrices (Figure 5.7). During this experiment it was clear to see that the α-SMA and cellular morphology expressed by normal scar cells was homogenous throughout the different matrices. In addition, within certain areas of anchored collagen matrices the cells had begun to line-up closely along side one another (Figure 5.7). Cells within both anchored collagen and fibrin matrices appeared taut and healthy. Within the contractile fibrin matrices cells expressed less α-SMA, the cells also appeared smaller but still healthy, well spread out, and linked together (Figure 5.7). Within contractile collagen matrices however, the normal scar cells appeared shrunken, very fragmented and unhealthy; with cell fragments also staining positive for α-SMA. These cells also appear separate from each other, exhibiting no obvious cell-cell contacts (Figure 5.7).

Keloid Scar Cells

By comparison to normal scar cells, α-SMA staining of keloid scar cell-seeded collagen matrices were vastly different (Figure 5.8). In anchored collagen matrices very few cells stained positive for α-SMA. Of those keloid scar cells that did stain positive for α-SMA, the staining was considerably less prominent and definite as that seen in anchored collagen matrices seeded with normal scar fibroblasts (Figure 5.5); requiring higher exposure times for clear image capture. Within the contractile collagen matrices there was virtually no α-SMA staining (Figure 5.8).

α-SMA staining of normal scar cells in collagen gels

Anchored



Contractile

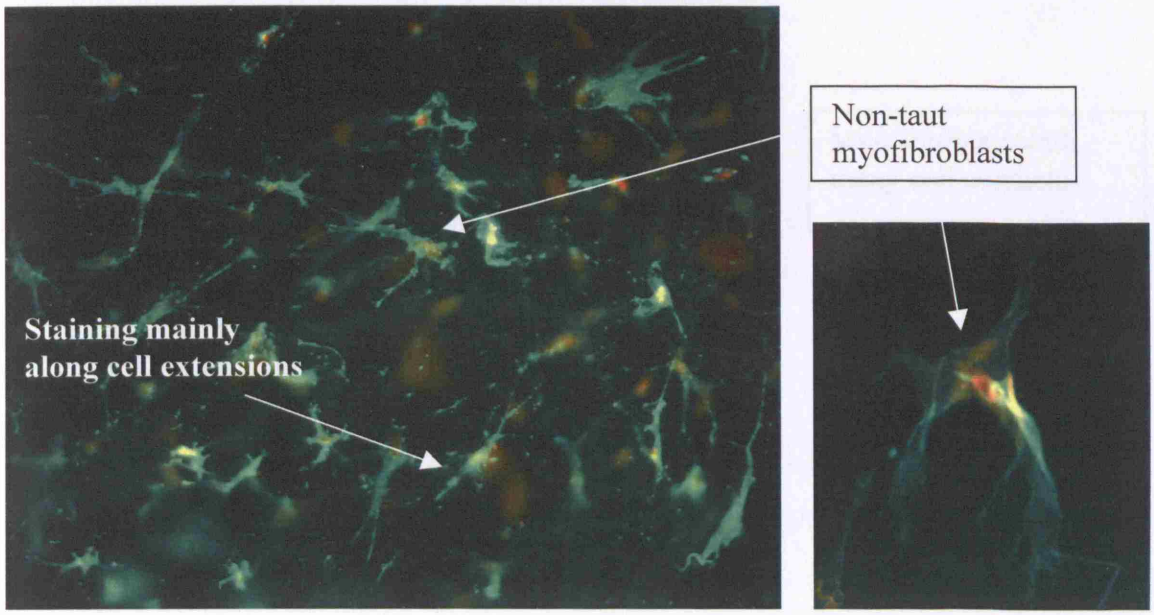
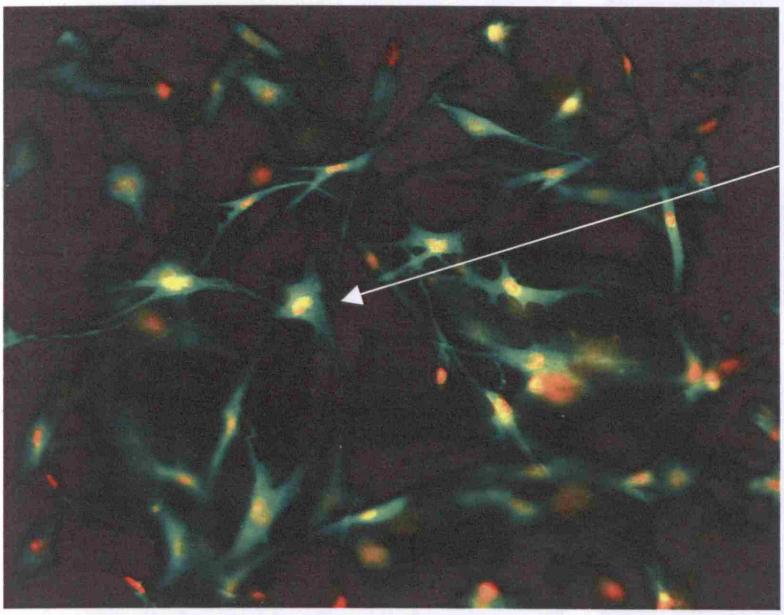


Figure 5.5 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of normal scar fibroblasts in collagen matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM. This experiment was carried out with n=6 normal scar cell strains. (x200 Mag).

α-SMA staining of normal scar cells in fibrin matrices

Anchored



Contractile

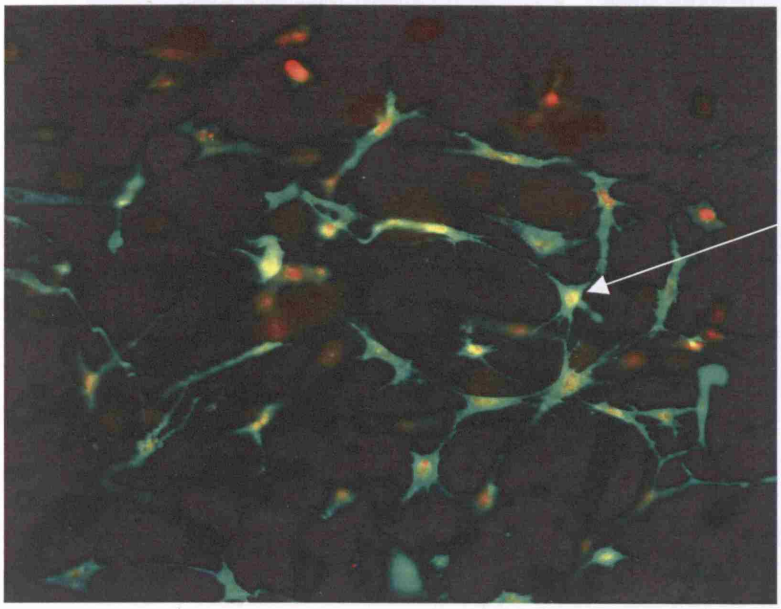


Figure 5.6 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of normal scar fibroblasts in fibrin matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM. This experiment was carried out with n=6 normal scar cell strains. (x200 Mag).

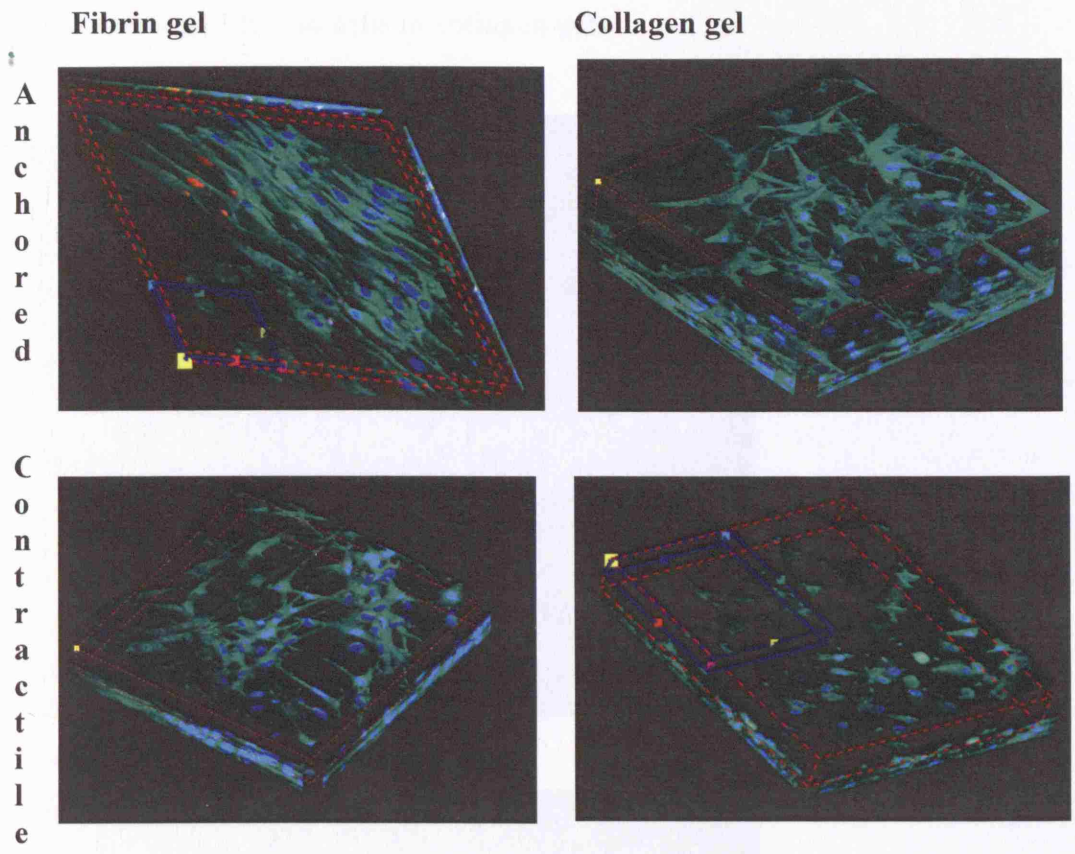
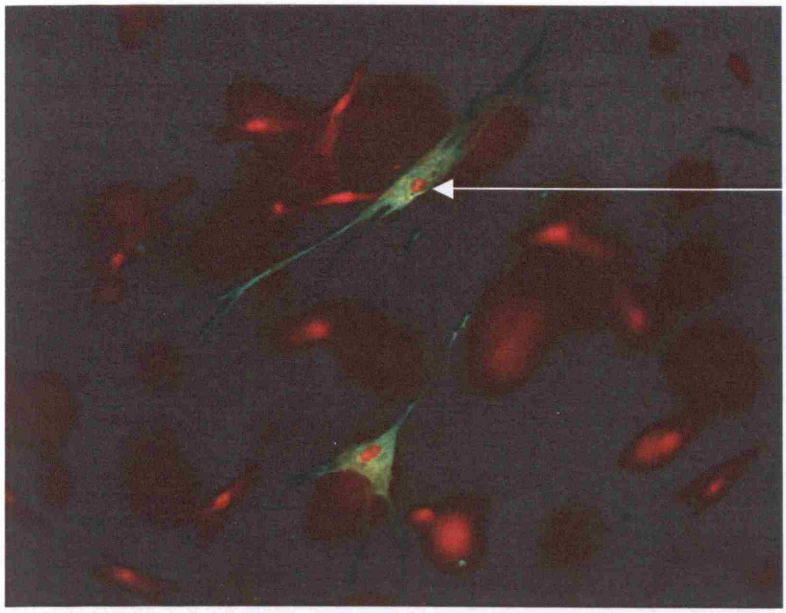


Figure 5.7 Typical confocal staining of α-SMA-stained myofibroblasts in day-7 anchored and contractile collagen and fibrin matrices seeded with normal scar-derived fibroblasts. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were fixed in 10% formal saline overnight, and then permeabilised with 0.1% Triton for 1hr before staining. FITC-conjugated α-SMA (green) and DAPI nuclear counter staining (blue) was used. This result is typical of n=4 normal scar cell strains.

Figure 5.9 illustrates α-SMA staining of keloid scar cells cultured within fibrin matrices. Within anchored fibrin matrices there was clear and consistent positive α-SMA staining. The cells that expressed α-SMA appeared much more elongated and spindle-like than normal scar fibroblasts within anchored fibrin matrices, with less prominent cell bodies (Figure 5.9). Nevertheless, the keloid scar cells appeared healthy and had many intercellular links. Within the contractile fibrin matrices the keloid scar cells were similar in appearance to that of normal scar cells within contractile fibrin matrices (Figure 5.6). However, keloid scar cells exhibited weaker staining for α-SMA than normal scar cells. Keloid scar cells were stellate in appearance, closely linked together through their cellular extensions and appeared healthy (Figure 5.9).

α-SMA staining of keloid cells in collagen gels

Anchored



Contractile

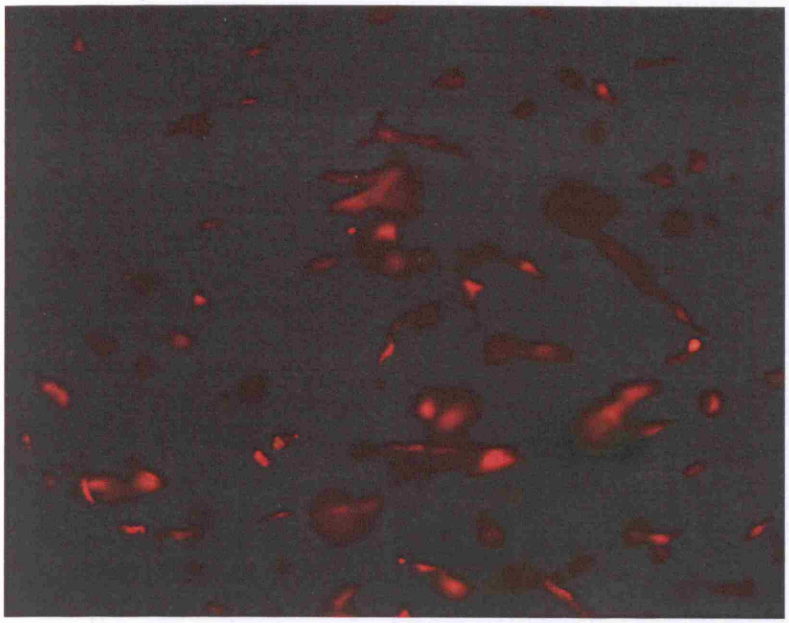
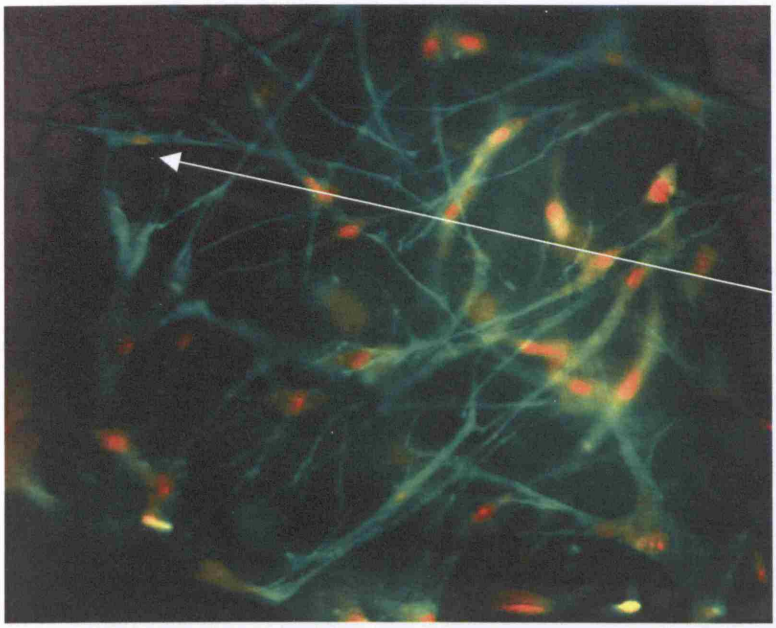


Figure 5.8 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of keloid scar fibroblasts in collagen matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM. This experiment was carried out with n=6 keloid scar cell strains. (x200 Mag).

α-SMA staining of keloid fibroblasts in fibrin matrices

Anchored



Contractile

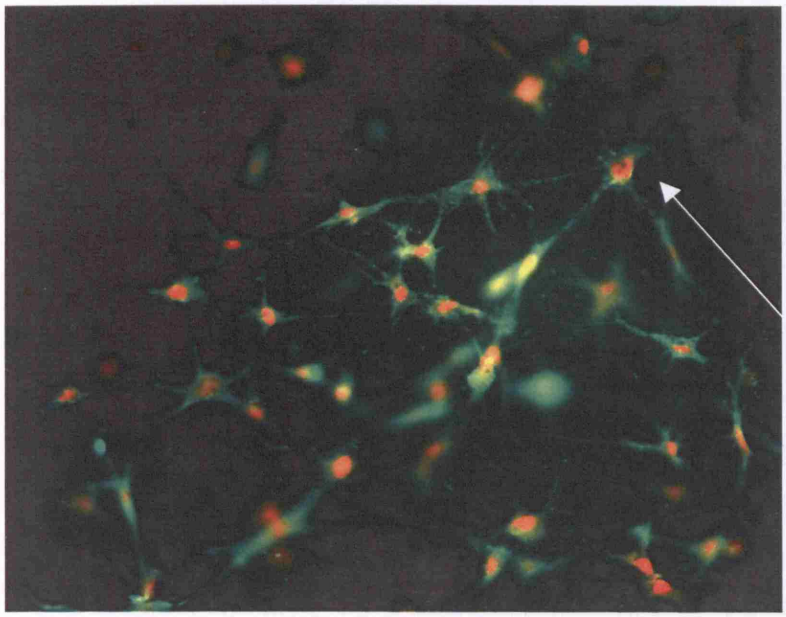


Figure 5.9 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of keloid scar fibroblasts in fibrin matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM. This experiment was carried out with n=6 keloid scar cell strains. (x200 Mag).

Taken together, these results indicate that in collagen matrices, the presence of myofibroblasts does correlate with whether apoptosis can be induced. Results from fibrin gels imply that the myofibroblast phenotype together with physical/mechanical contraction is not sufficient to induce apoptosis and that collagen is essentially involved with the apoptosis.

5.2.2 Can Keloid Scar Fibroblasts be Induced to Differentiate into Myofibroblasts in 3-D Collagen Gels?

Keloid scar cells and normal scar cells (control) were seeded into anchored collagen matrices and maintained for 7-days in 1% NGM + TGF-β1. *In situ* staining for α-SMA was carried out as before with FITC fluorescence microscopy. Micrographs are presented in Figures 5.10 and 5.11. Quantification of the number of myofibroblasts was not assessed due to the difficulties encountered by the 3-D nature of the matrix the cells were cultured in. Instead, the experiments only assessed whether the fibroblasts seeded into the matrices had differentiated into myofibroblasts or not.

Within anchored collagen matrices in the presence of TGF-β1, the appearance of normal scar cells was relatively unaffected compared to that in the absence of TGF-β1, with the majority strongly expressing α-SMA (Figure 5.10). Cell bodies appeared enlarged with some cells expressing numerous cell processes protruding from the cell body in a mainly bipolar orientation. In contrast to normal scar cells maintained in the absence of TGF-β1 however, there was great variety in the extent of α-SMA expressed by the cells treated with TGF-β1. In addition, random areas of the matrix contained myofibroblasts, which appeared to have severed linkages with neighbouring cells and the matrix itself; with the cells appearing loosely held within the matrix, fragmented and sometimes stellate (Figure 5.10). The appearance of these cells was similar to that of the cells within contractile collagen gels.

In the presence of TGF-β1 keloid scar fibroblasts cultured in anchored collagen matrices also stained positive for α-SMA (Figure 5.11). These cells were mainly bipolar and stretched along lines of tension however; the staining pattern within the cells appeared fragmented. There appeared to be no definite outline to the cells' shape, as if the

organisation of the α-SMA was not continuous along the stress fibres within the cell. This irregular staining pattern was seen throughout the collagen matrix (Figure 5.12).

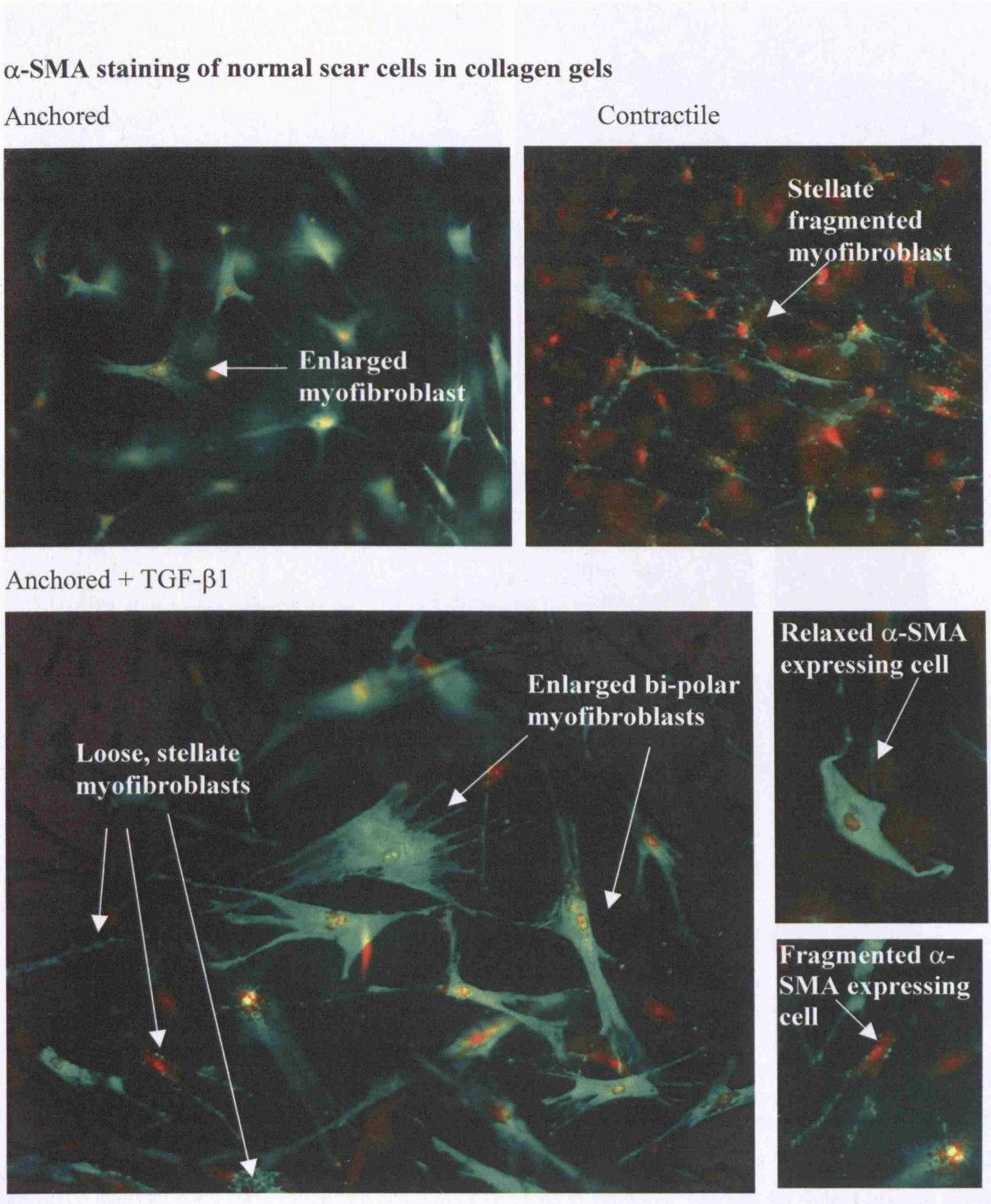
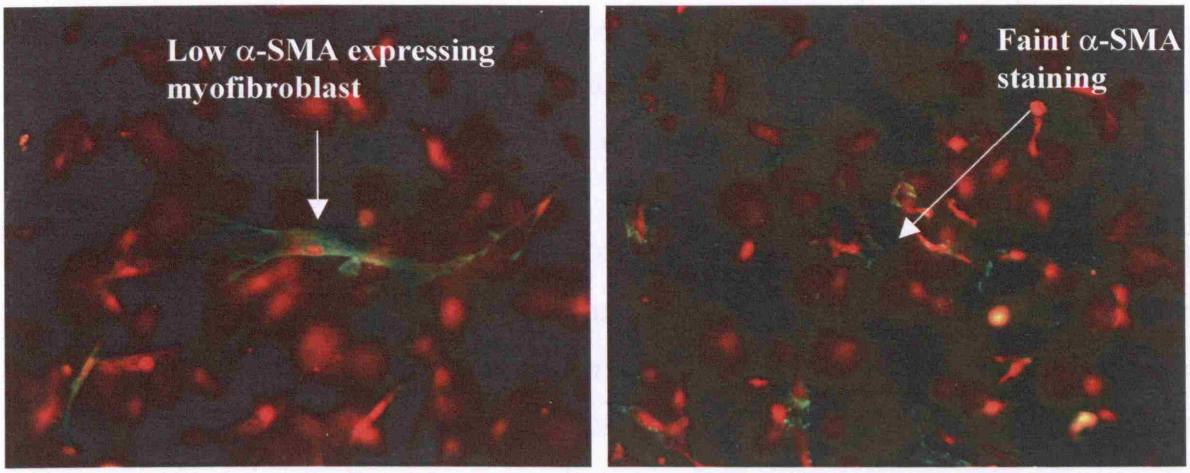


Figure 5.10 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of normal scar fibroblasts in collagen matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM with or without 2ng/ml TGF-β1. This experiment was carried out with n=6 normal scar cell strains. (x200 Mag).

α-SMA staining of keloid cells in collagen gels

Anchored

Contractile



Anchored + TGF-β1

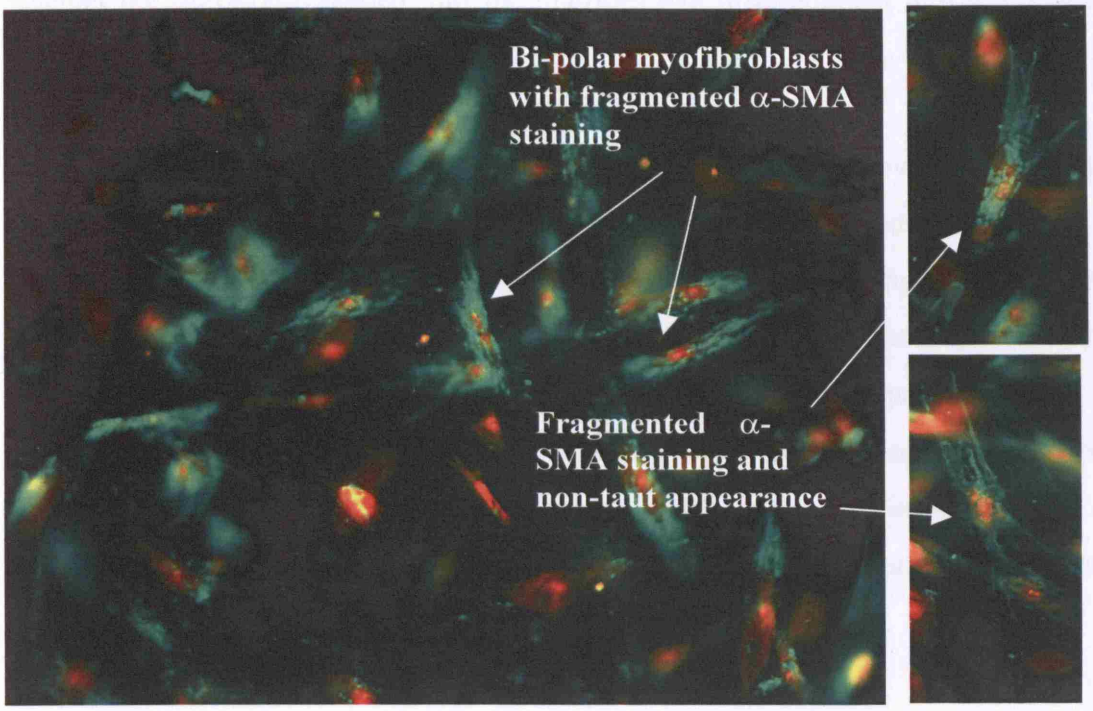


Figure 5.11 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of keloid scar fibroblasts in collagen matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM with or without 2ng/ml TGF-β1. This experiment was carried out with n=6 keloid scar cell strains. (x200 Mag).

These results show that keloid scar-derived fibroblasts can indeed be induced to differentiate into myofibroblasts by TGF-β1 (Figure 5.11). What was very interesting to note however, was that in anchored collagen matrices in the presence of TGF-β1, cells derived from both normal scar and keloid scar showed occasional weaker stained loose, fragmented cells; similar to that seen in contractile collagen gels.

In order to determine if this cell fragmentation effect of TGF-β1 was restricted to collagen matrices, α-SMA staining of normal scar and keloid scar cells maintained in anchored fibrin matrices treated with TGF-β1 was carried out. As with collagen gels, quantification of the number of myofibroblasts was not assessed due to the difficulties encountered by the 3-D nature of the matrix the cells were cultured in. Instead, the experiments only assessed whether the fibroblasts seeded into the matrices had differentiated into myofibroblasts or not.

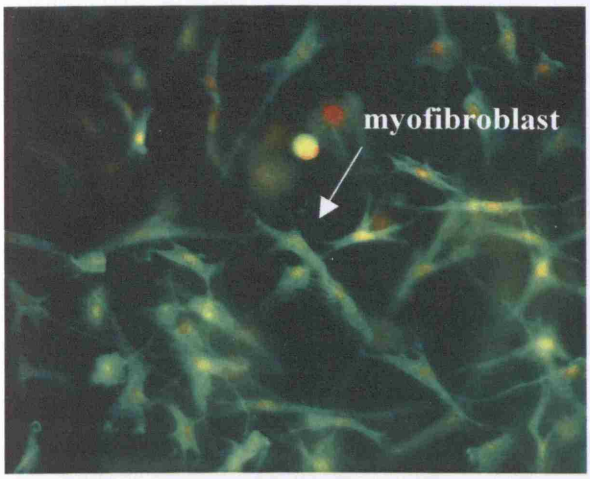
Results demonstrated that with the addition of TGF-β1 to the growth medium of normal scar cells cultured within anchored fibrin matrices, cells stained strongly for α-SMA (Figure 5.12). The appearance of normal scar cells was relatively homogenous throughout the matrix. Normal scar cells appeared stretched and clearly bipolar (Figure 5.12). Only a small number of cells in anchored fibrin matrices in the presence of TGF-β1 showed increased α-SMA staining around the nucleus of the cell and numerous cell processes extending from the enlarged cell bodies (Figure 5.10); like those seen in anchored collagen gels in the presence of TGF-β1. There was no obvious loose appearance, fragmentation or rounding-up of the cells.

Keloid scar cells cultured within anchored fibrin matrices, in the presence of TGF-β1 also showed increased α-SMA expression compared to that in the absence of TGF-β1 (Figure 5.13). In the presence of TGF-β1 keloid scar cells appeared more compact and stained positive for α-SMA (Figure 5.13). This was in contrast to the very linear and more elongated appearance of normal scar cells seeded within anchored fibrin matrices in the presence of TGF-β1 (Figure 5.12). The keloid scar cells again appeared bipolar and firmly held within the matrix environment. No signs of cells rounding-up, fragmentation or loosening were apparent. This staining pattern was similar throughout the fibrin matrix,

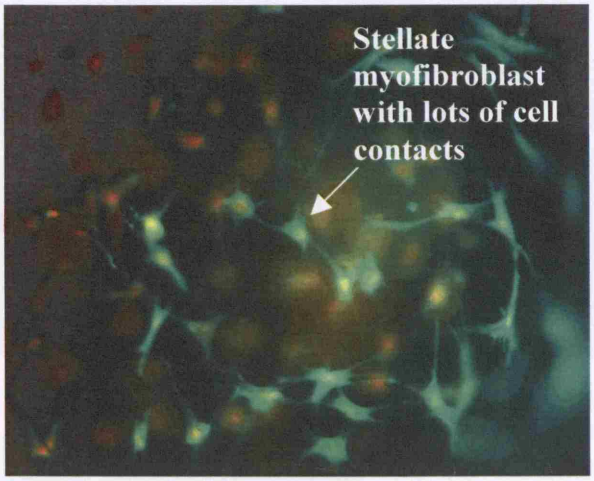
and unlike in anchored collagen matrices in the presence of TGF-β1 the staining pattern within the cells appeared continuous rather than fragmented (Figure 5.13).

α-SMA staining of normal scar cells in fibrin matrices

Anchored



Contractile



Anchored + TGF-β1

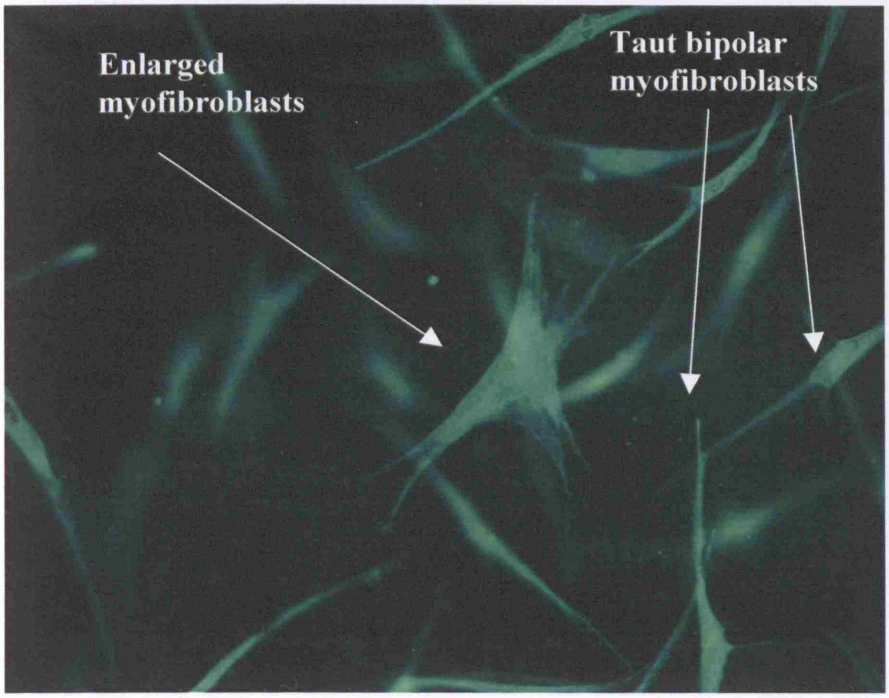
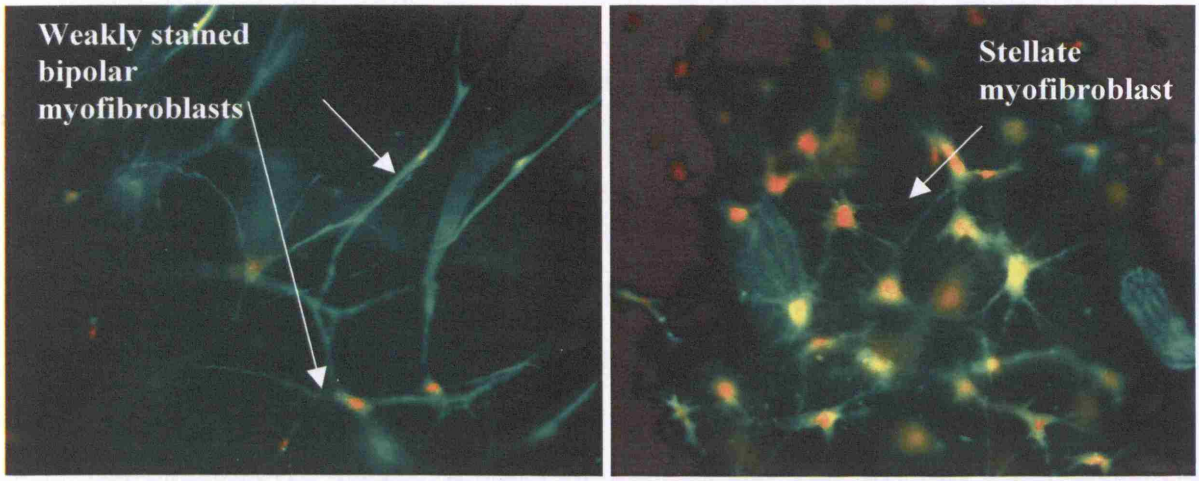


Figure 5.12 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of normal scar fibroblasts in fibrin matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM with or without 2ng/ml TGF-β1. This experiment was carried out with n=6 normal scar cell strains. (x200 Mag).

α-SMA staining of keloid fibroblasts in fibrin matrices

Anchored

Contractile



Anchored + TGF-β1

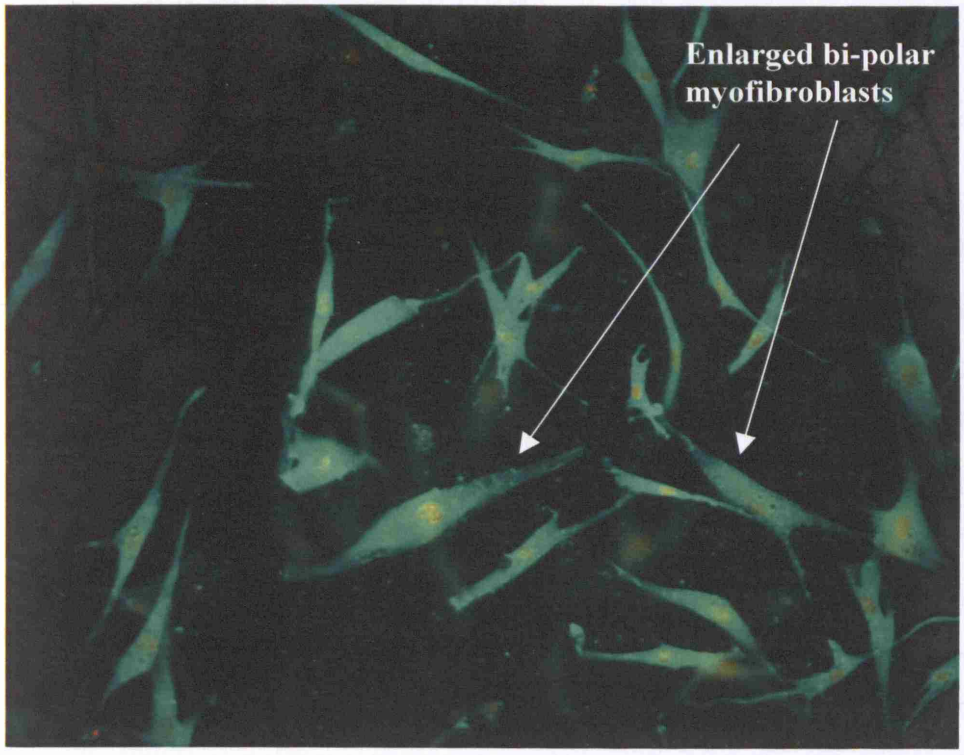


Figure 5.13 Typical fluorescent α-smooth muscle actin staining (green) with propidium iodide counterstaining (red) of keloid scar fibroblasts in fibrin matrices. The cells were either maintained in anchored matrices for 7-days preceding fixation or cultured in anchored matrices for 4-days prior to allowing the cells to contract the matrices for 3-days. Cells were cultured in 1% NGM with or without 2ng/ml TGF-β1. This experiment was carried out with n=6 keloid scar cell strains. (x200 Mag).

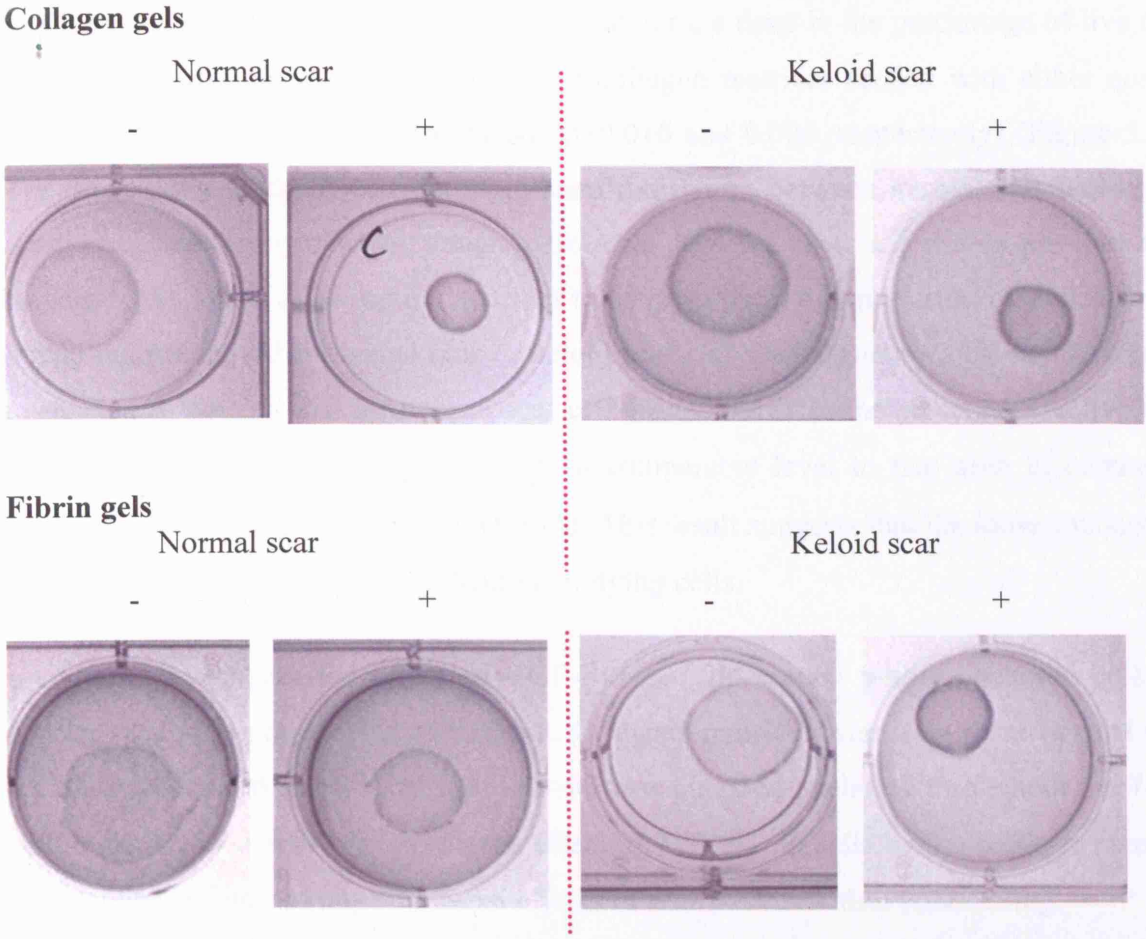


Figure 5.14 Typical degree of contraction produced by normal scar and keloid scar fibroblasts seeded within collagen and fibrin matrices. Cells were maintained in anchored matrices for a period of 4-days before releasing matrices to allow contraction for a further 3-days. Cells were cultured in 1% NGM with (+)\or without (-) the addition of TGF-β1at 2ng/ml. This result is representative of n=5 normal scar and keloid scar cell strains.

Table 5.1 Statistical analysis of fibrin and collagen matrix contraction induced by normal scar and keloid scar fibroblasts in the presence of TGF-β1 at 2ng/ml to that induced in the absence of TGF-β1. Data represents the percentage change in matrix circumference from that of the tissue culture well (original gel circumference) by day-7 of matrix culture. Raw data was analysed by T-test comparing the degree of contraction induced in 1% NGM to that induced in 1% NGM+TGF-β1. *P = <0.05.

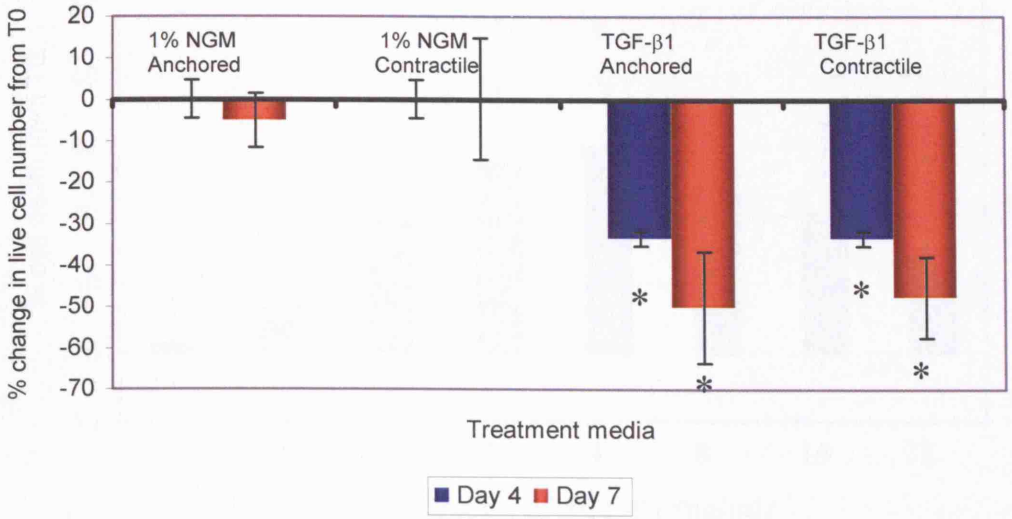
Mean % Change in Gel Circumference				
Matrix Type	**Cell Type**	**1% NGM**	**1% NGM + TGF-β1**	**P-Value (1% NGM vs 1% NGM+TGF-β1)**
Collagen	Normal Scar	47.08 ± 3.0	60.47 ± 4.5	0.013*
Collagen	Keloid Scar	47.94 ± 2.6	64.01 ± 4.2	0.005*
Fibrin	Normal Scar	45.30 ± 1.8	58.8 ± 2.9	0.003*
Fibrin	Keloid Scar	48.06 ± 3.8	62.04 ± 1.5	0.004*

With the addition of TGF-β1 to the culture medium, a drop in the percentage of live cells occurred surprisingly at day-4 in anchored collagen matrices seeded with either normal scar- and keloid scar-derived fibroblasts ($p=0.016$ and 0.004, respectively) (Figure 5.15). The degree of cell death was not significantly different between normal scar and keloid scar seeded collagen matrices, ranging from 30-40% for $n=4$ cell strains per scar type (Figure 5.15). When allowing the matrices to contract there was no further significant drop in cell number in either normal scar or keloid scar cell-seeded matrices by day-7. What is interesting to note, is that for normal scar cell-seeded matrices the percentage of live cells in TGF-β1-treated anchored gels falls to a comparable level to that seen in contractile collagen matrices in the absence of TGF-β1. This result suggests that the loose-rounded-up cells seen in section 5.2.2 were indicative of dying cells.

To further investigate the effect of TGF-β1 on cells seeded within anchored collagen matrices a titration assay was carried out. Collagen matrices were set-up and seeded with normal scar-derived fibroblasts. Matrices were maintained anchored throughout the 7-day culture period to specifically assess the effect of TGF-β1 on cells within collagen matrices alone, in the absence of any additional effects of matrix contraction/remodelling.

TGF-β1 was assessed at titrated concentrations from 0-32ng/ml in 1% NGM. Figure 5.16 illustrates the percentage of cell death induced by TGF-β1. Within collagen matrices this appears to peak at 2, 4 and 8ng/ml, where a significant ($p=0.026$, 0.007 and 0.018, respectively) induction of cell death occurs (~ 30%) in comparison to that detected in the absence of TGF-β1. The other titrated concentrations tested did not elicit a statistically significant induction of cell death in collagen matrices. These results show that the effect of TGF-β1 is dose-dependant. It was decided that for economic reasons, the lowest of the TGF-β1 concentrations (2ng/ml) that induced a significant amount of cell death would be used for all further experiments.

Keloid scar



Normal scar

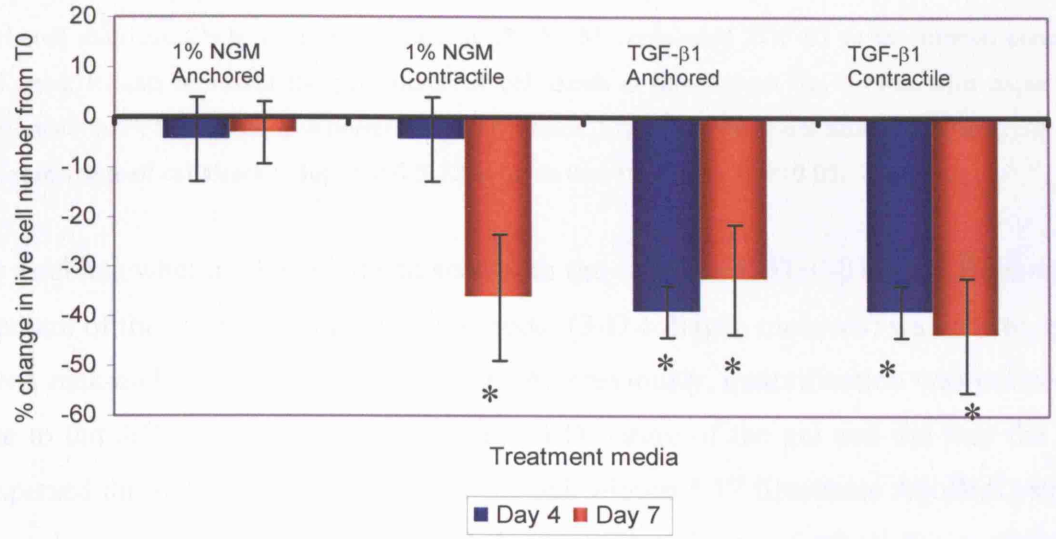


Figure 5.15 Fibroblasts derived from normal scar or keloid scar were seeded into 3-D collagen gels. At day-0 (T0-24hrs after seeding gels and prior to adding growth media), 4 and 7 live cell number was assessed in anchored (stressed) and contractile (stress-relaxed) matrices. Cells were maintained in 1% NGM with or without 2ng/ml TGF-β1. Results represent the percentage change in cell number from T0. This experiment was performed in triplicate with n=4 for each scar type. Error bars represent SD. T-test analysis compared the percentage change in live cell number after each gel treatment to the that in anchored gels supplemented with 1% NGM only (day-4 vs day-4 and day-7 vs day-7). *P=<0.05.

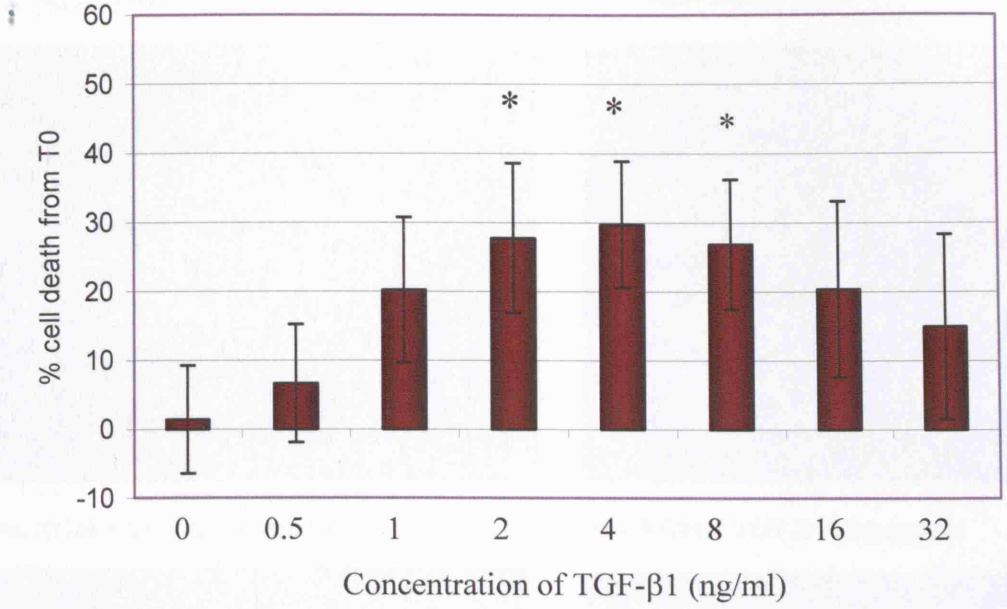


Figure 5.16 Fibroblasts derived from normal scar were seeded into anchored collagen matrices. At day-0 (T0-24hrs after seeding gels and prior to adding growth media), and day-7 cell number was assessed in the anchored matrices. Cells were maintained in 1% NGM containing TGF-β1 at the titrated concentrations indicated. Results represent the percentage of cell death at day-7 from day-0 (T0). This experiment was performed in triplicate with n=4 normal scar cell strains. Error bars represent SEM. T-test analysis compared the percentage of cell death induced at 0.5-32ng/ml vs that at 0ng/ml. *P=<0.05.

To establish whether the cell death seen with the addition of TGF-β1 to the minimal culture medium of the *in vitro* wound healing model (3-D collagen matrices) was due to apoptosis, DNA nick-end labelling was carried out. As previously, quantification was not carried out due to the difficulties encountered by the 3-D nature of the gel and the way the cells are dispersed through the many phases of the gel. Figure 5.17 illustrates ApoBrdU staining of normal scar and keloid scar cell seeded anchored collagen matrices. No apoptotic nuclei was detected in anchored matrices where cells were maintained in 1% NGM alone, however numerous apoptotic nuclei was detected in matrices where cells were maintained in 1% NGM + TGF-β1 at 2ng/ml at 7-days.

These results demonstrate that the treatment of scar cells with TGF-β1 induces apoptosis in collagen gels in the absence of gel contraction. To find out whether the apoptosis induced by TGF-β1 is specific to a collagen matrix; like collagen contraction-induced apoptosis, the effect of TGF-β1 was studied with cells seeded in fibrin matrices.

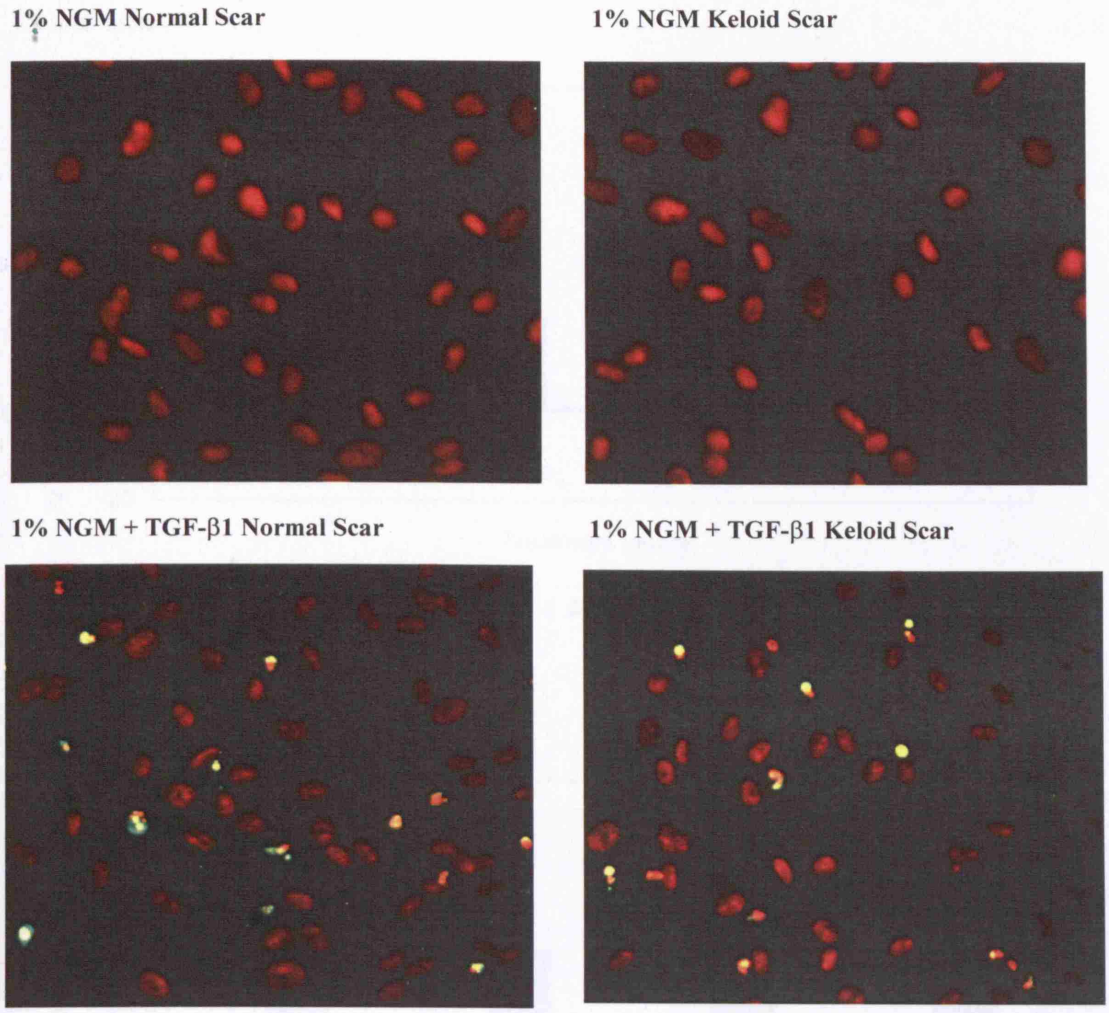
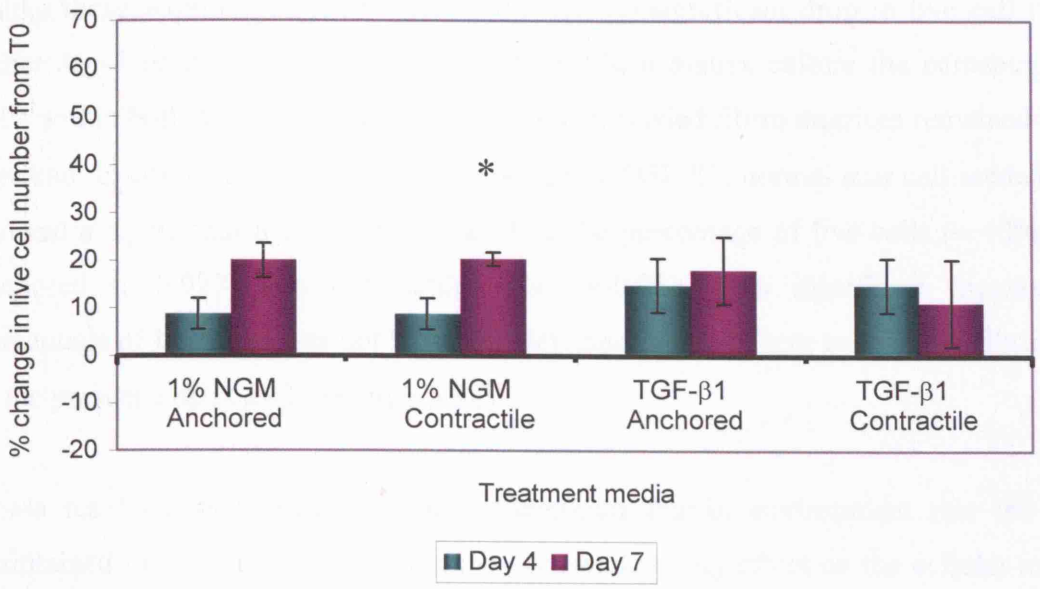


Figure 5.17 Representative micrographs of terminal deoxynucleotidyl transferase end-labelling of apoptotic nuclei (yellow/green) with red (propidium iodide) counter-staining of normal scar and keloid scar fibroblasts seeded within anchored collagen matrices for 7-days. The cells were cultured in 1% NGM with or without TGF-β1 at 2ng/ml. This experiment was carried out in triplicate with n=4 normal scar and keloid scar cell strains. (x200 Mag).

5.2.5 Does TGF-β1 Affect the Viability of Cells within Fibrin Matrices?

When studying the effect of TGF-β1 on scar fibroblasts seeded within fibrin matrices a very different response was seen (Figure 5.18). In both normal scar and keloid scar cell seeded matrices (as shown previously in Chapter 3) in the absence of TGF-β1, there was no significant change in the percentage of live cells within anchored matrices over the 7-day time course. However, on releasing fibrin matrices at day-4 there was a significant increase in the percentage of live cells within normal scar and keloid scar cell-seeded contractile matrices by day-7 ($p=0.025$ and $p= 0.031$, correspondingly) compared to that at T0, between 20-35%.

Keloid scar



Normal scar

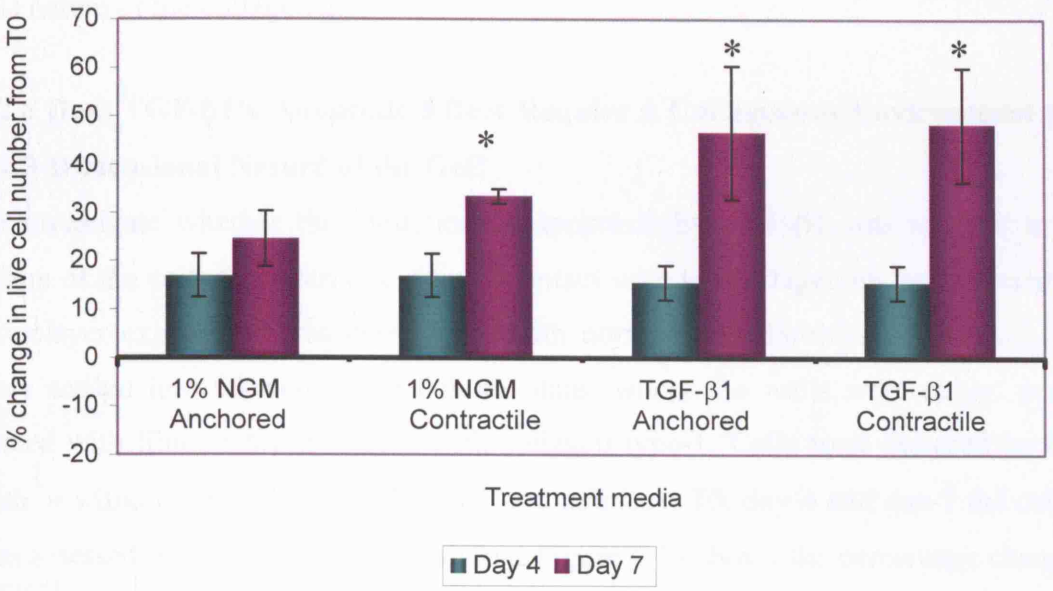


Figure 5.18 Fibroblasts derived from normal scar or keloid scar were seeded into 3-D fibrin matrices. At day-0 (24hrs after seeding gels and prior to adding growth media), -4 and -7 live cell number was assessed in anchored and contractile matrices. Cells were maintained in 1% NGM with or without 2ng/ml TGF-β. Results represent the percentage change in cell number from T0. The experiment was performed in triplicate with n=4 for each scar type. Error bars represent SD. T-test analysis compared the percentage change in live cell number after each gel treatment to that in anchored gels supplemented with 1% NGM only (day-4 vs day-4 and day-7 vs day-7). *P=<0.05.

In the presence of TGF-β1, cells from either scar type maintained within fibrin matrices, unlike those within collagen matrices, showed no significant drop in live cell number at either day-4 or day-7. At day-4 of anchored fibrin matrix culture the percentage of live cells within both normal scar and keloid scar cell seeded fibrin matrices remained relatively constant. In contrast, by day-7 in the presence of TGF-β1, normal scar cell seeded matrices showed a significant increase from day-4 in the percentage of live cells (~ 40%), in both anchored ($p=0.027$) and contractile gels ($p=0.03$). This significant increase in the percentage of live cells was not however, detected in keloid scar cell seeded fibrin matrices in the presence of TGF-β1 (Figure 5.18).

These results demonstrate that the extracellular matrix environment that the cells are maintained in can have enormous and indeed contrasting effect on the cellular response to TGF-β1. The apoptotic effect of TGF-β1 appears to be specific to a collagenous environment. It is not known however, whether this apoptotic effect is also specific to the 3-D nature of the collagen gel.

5.2.6 Does TGF-β1's Apoptotic Effect Require a Collagenous Environment Alone or the 3-Dimensional Nature of the Gel?

To investigate whether the induction of apoptosis by TGF-β1 was specific to the 3-D nature of the collagen matrix or simply contact with the collagenous environment itself, a monolayer experiment was carried out with normal scar fibroblasts (Figure 5.19). Cells were seeded into 96-well tissue culture plates where the wells were either: non-coated, coated with fibronectin or coated with collagen type-1. Cells were cultured in 1% NGM with or without the addition of TGF-β1 at 2ng/ml. At T0, day-4 and day-7 the cell number was assessed with Crystal Violet staining. Figure 5.19 shows the percentage change in cell number from time zero (T0). Although, there appears to be a varied response to the growth environments (± 20% change in cell number from time zero) there is no significant change in live cell number for fibronectin-coated or non-coated monolayers in the absence or the presence of TGF-β1 (Figure 5.19). However, surprisingly the collagen-coated monolayers did show a significant ($p=0.029$) increase (~ 40%) in the percentage of live cells by day-7 from T0 in the presence of TGF-β1 (Figure 5.19). This was not true of cells cultured on collagen coated monolayers in the absence of TGF-β1 however, even though there was ~20% increase in live cell number. Importantly though, the significant change in cell

number seen with fibroblasts cultured on collagen monolayers in the presence of TGF-β1 was that of increasing cell number, not cell death, as is detected in 3-D collagen matrices.

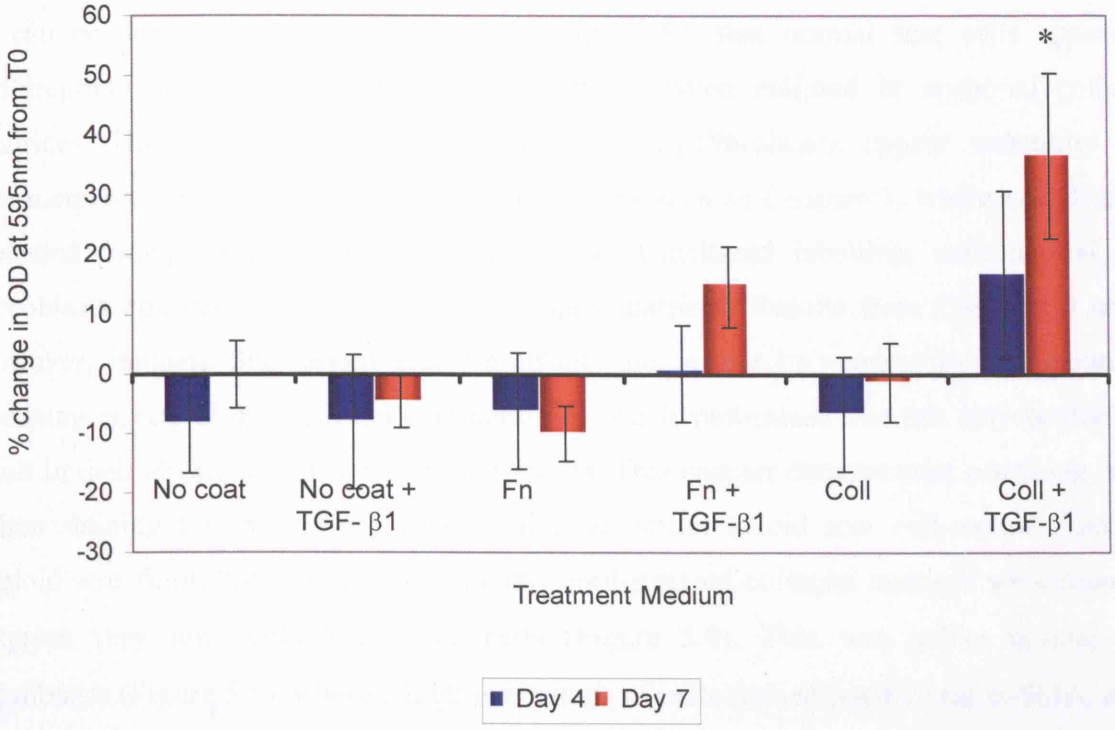


Figure 5.19 The effect of TGF-β1 on monolayer cultured fibroblasts derived from normal scar. OD at 595nm represents cell number. 96-well tissue culture plates were either coated with fibronectin (Fn) at 10μg/ml, collagen (Coll) at 10μg/ml or with no coating. Cells were seeded and maintained in 1% NGM with or without 2ng/ml TGF-β1. At day-0 (24hrs after seeding cells), -4 and -7 cell number was assessed using Crystal Violet staining. Results represent the percentage change in cell number from T0, analysed by T-test analysis. The experiment was performed in triplicate with n=5 normal scar cell strains. Error bars represent SEM. *P=<0.05.

5.3 Discussion

The main initial aim of this chapter was to determine whether collagen contraction-induced apoptosis only takes place if the cells are of a myofibroblast phenotype. Although results gained in this chapter provide some circumstantial evidence for this, the main finding is a novel and very exciting role for active-TGF-β1, in its ability to induce apoptosis of both normal scar and keloid scar fibroblasts. As with the apoptosis that is induced during collagen contraction, the apoptosis induced by TGF-β1 is also specific to a 3-D collagenous matrix. It is possible that this is a discovery of a previously unsuspected role of

TGF-β1 in the clearance of granulation tissue cells. Results gained from *in situ* cell staining revealed that this apoptosis may require the myofibroblast phenotype.

5.3.1 Does the Sensitivity to Apoptosis Depend on the Myofibroblast Phenotype?

It can be seen in the micrographs from Figure 5.5 that normal scar cells appear to differentiate into healthy looking myofibroblasts when cultured in anchored collagen matrices. However, after gel contraction, the myofibroblasts appear unhealthy and fragmented. This result correlates with the results seen in Chapter 3, where cell death is detected through viable cell counting and DNA-nick-end labelling, with normal scar fibroblasts cultured within contractile collagen matrices. Results from Chapters 3 and 4 however, indicate that keloid scar fibroblasts are unable to respond to the apoptosis-inducing effects of collagen gel contraction. It was hypothesised that this may be due to a fault in their ability to turn into myofibroblasts. This chapter demonstrates a striking result when staining for the myofibroblast cell type within keloid scar cell-seeded matrices. Keloid scar fibroblasts cultured within anchored-stressed collagen matrices were found to express very few α-SMA positive cells (Figure 5.8). This was unlike normal scar fibroblasts (Figure 5.5), where a high percentage of cells stained positive for α-SMA. After collagen gel contraction, keloid scar cells expressed very little α-SMA staining (Figure 5.8).

Within fibrin gels normal scars cells expressed high levels of α-SMA and the myofibroblasts appeared very healthy. Surprisingly, keloid scar fibroblasts maintained within anchored fibrin matrices (Figure 5.9) were also capable of expressing α-SMA. These myofibroblasts appeared very different morphologically to that of normal scar cells being spindle-like but still relatively healthy. After fibrin gel contraction (Figure 5.6 and 5.9), the morphology and number of cells expressing α-SMA were very similar between the two scar types. The cells appeared much smaller and stellate in morphology. Compared to the morphology of the cells in contractile collagen gels, both scar cell types showed a much healthier morphology. The cells appeared less dendritic and more retracted and highly linked together. Results from Chapter 3 demonstrate that cells seeded within contractile fibrin gels are not signalled to undergo any type of cell death, which is consistent with the morphological staining. These results indicate that a collagenous environment is specifically required for apoptosis and that the myofibroblast phenotype

along with 3-D gel contraction is not sufficient to induce apoptosis. These results are the first of their kind to discuss the effect of 3-D collagen and fibrin matrix culture in low serum conditions on the phenotypic and morphological features of normal scar and keloid scar fibroblasts. It is therefore difficult to discuss these results in reference to other authors, however these results may be extremely important in the progression of research into the apoptosis cues that lead to the termination of wound healing events.

5.3.2 Can Keloid Scar Fibroblasts be Induced to Differentiate into Myofibroblasts?

In the presence of TGF-β1, cells derived from normal scar and keloid scar were able to differentiate into prominent myofibroblasts when cultured in anchored collagen and fibrin gels (Figure 5.10-5.13). The cells expressed increased amounts of α-SMA, having a bipolar and enlarged morphology. This result confirms that keloid scar-derived fibroblasts do have the ability to differentiate into myofibroblasts expressing α-SMA.

Unexpectedly it was seen within anchored collagen gels seeded with normal scar cells in the presence of TGF-β1 that some cells appeared stellate and loose, possibly having lost cell-contacts. Staining was also fragmented within some cells. The α-SMA staining within keloid scar fibroblasts in collagen gels was also very fragmented. This was not seen with TGF-β1 treatment of cells in fibrin gels. This led to the suggestion that both types of scar cells were possibly responding to TGF-β1 in a similar way to that which occurs with normal scar cells during collagen-contraction. It was hypothesised that these were signs of cell death.

5.3.3 TGF-β1 Induction of Apoptosis in Collagen Gels in the Absence of Contraction

It was the intention of this chapter to use TGF-β1-induction of myofibroblast differentiation of keloid scar cells, to explore whether the myofibroblast phenotype is required for collagen contraction-induced apoptosis. Results from Figure 5.15 show that although keloid scar cells were able to undergo apoptosis in contractile collagen gels in the presence of TGF-β1, and to an equivalent level as that seen with normal scar cell-seeded collagen gels, they also underwent a significant amount of apoptosis in anchored collagen gels in the presence of TGF-β1 ($p=0.016$ and 0.004, respectively). This phenomenon was also seen with normal scar cells and was not detected with cells cultured in fibrin gels. These results correlate with results from morphological staining, where signs of cell death

were detected in cells cultured within anchored collagen gels in the presence of TGF-β1; whereas cells maintained within anchored fibrin matrices appeared very healthy. Thus TGF-β1 appears to by-pass the requirement for collagen contraction in the apoptosis induction of cells in 3-D collagen gels.

TGF-β1 was found to significantly increase the degree of gel contraction by normal scar and keloid scar cells in both matrix types. This increased contraction was found to be equivalent in contractile collagen and fibrin matrices, irrespective of cell type. Importantly however, the degree of contraction had no correlation to the amount of apoptosis taking place within the matrix (discussed further in Chapter 7).

Interestingly, the apoptosis that occurred in the presence of TGF-β1 was not further increased on gel contraction. This perhaps signifies that there is no additional apoptosis mechanism involved. This may imply that the mechanism of apoptosis induction via TGF-β1 and collagen contraction may be through similar pathways.

If this assumption is correct then these results may indicate that keloid scar cells in the presence of TGF-β1 are capable of responding to the cues of collagen contraction-induced apoptosis. Whether this is simply through keloid scar cells differentiating into myofibroblasts within collagen gels and being able to respond to apoptosis cues or, through the ability of keloid scar myofibroblasts to produce apoptosis cues in the presence of TGF-β1 is not yet known.

5.3.4 Does the Apoptosis Induced by TGF-β1 Rely Simply on a Collagenous Environment?

An investigation was carried out to assess whether the 3-D nature of the collagen gel was needed for TGF-β1-induced apoptosis, or simply cell-matrix contact with collagen. Results determined that TGF-β1 had no significant effect on monolayer cultured normal scar cells maintained on non-coated or fibronectin-coated plastic. Normal scar fibroblasts cultured on collagen-coated plastic however, were induced to undergo a significant ($p=0.029$) degree of cell proliferation on treatment with TGF-β1. Although there is no evidence in the literature with regards to the effect of TGF-β1 on fibroblasts cultured on collagen-coated

monolayers, Kottler *et al.* (2005) shows that TGF-β1 stimulates cell proliferation of human Tenon's capsule fibroblasts in monolayer culture using the WST-1 colorimetric assay.

This failure of TGF-β1 to induce apoptosis of cells cultured on collagen-coated plastic implies that simple cell-matrix contact is not enough; instead something essential to the 3-D nature of the collagen gels is required for apoptosis induction. These results also further demonstrate the pleiotropic effects of TGF-β1, its role as a mediator of many effects controlled through cell morphology, growth environment, stress and many other factors. This is in agreement with Grotendorst *et al.* (2004) who found that fibroblast proliferation and differentiation is controlled by TGF-β1, but through exclusive signalling pathways utilising different sub-sets of growth factors.

These results demonstrate that TGF-β1 may act through separate pathways in order to signal cell differentiation and cell apoptosis. This may potentially rely on the cells microenvironment, cell phenotype, cell-tension, integrin expression and growth factor availability. It is possible that for these reasons (which are more than likely the same reasons that no cell death occurs on fibrin-contraction) no cell death is induced in fibrin matrices in the presence of TGF-β1 with either normal scar or keloid scar cells.

5.3.5 Summary

These results therefore suggest that the apoptosis that takes place during matrix-contraction or with the addition of TGF-β1 is specific to the signalling that is able to take place when cells are cultured within a 3-dimensional collagen matrix. These factors may be a crucial control mechanism, preventing cell clearance from the wound site until the final phase of wound healing where the matrix becomes that of mainly collagen type I.

In addition, results from this chapter demonstrate that keloid scar fibroblasts have a fault in their ability to differentiate into myofibroblasts whilst cultured within anchored-stressed collagen matrices, as normal scar fibroblasts do. This may potentially be the reason keloid scar cells do not respond to collagen contraction-induced apoptosis. A possible reason for this is aberrant autocrine signalling of factors inducive to myofibroblast differentiation, which appears to be rectified by the addition of exogenous active TGF-β1. As discussed in section 5.1, myofibroblast differentiation may be crucial to the apoptosis detected during

collagen matrix contraction; either through successfully producing the apoptosis cues and/or the ability of cells to respond to the apoptosis cues. This finding illustrates a potential defect in the secretion or activation of growth factors secreted by keloid scar fibroblasts, possibly TGF-β1.

This finding nevertheless conflicts with the observation that activated fibroblasts and often myofibroblasts are commonly found in active keloid scars. A possible reason for the presence of myofibroblasts within keloid scars is through paracrine signalling from surrounding epithelial and lymphatic cells. Alongside the presence of cell differentiation factors however, there is potentially elevated growth factor and cytokine signalling, providing anti-apoptotic signals that may out-compete the presence of any pro-apoptotic signals. There is evidence in the literature for this phenomenon. Specifically, Messadi *et al.* (2004) found that keloid scar fibroblasts and tissues exhibit higher basal levels of various anti-apoptotic factors such as: TNF-receptor-associated factors (TRAF1, TRAF2, TNF-α), inhibitor of apoptosis protein (C-IAP-1) and NfκB-binding activity together with its targeted genes in comparison to that expressed by normal scars (section 1.7 further describes these apoptosis-related proteins). In another study by Funayama *et al.* (2003) keloid scar-derived keratinocytes were reported to significantly upregulate ERK and JNK phosphorylation and the expression of Bcl-2 and TGF-β1 in co-cultured fibroblasts derived from both keloid scar and normal dermis. A study by Sayah *et al.* (1999) also determined that apoptosis-related genes in keloid scar were under-expressed in comparison to that expressed by normal scars. In addition to the clear evidence for increased anti-apoptotic signalling, keloid scar fibroblasts also have a reduced growth factor requirement (Russell *et al.*, 1988). This could be attributed to increased growth factor receptor expression; indeed there is evidence in the literature for increased IGF-1, PDGF and TGF-β1 receptors (Haisa *et al.*, 1994; Yoshimoto *et al.*, 1999; Ohtsuru *et al.*, 2000; Chin *et al.*, 2000). Nevertheless, the results presented in this chapter identify a distinct fault with keloid scar-derived fibroblasts themselves.

Chapter 6 further investigates the mechanism of apoptosis induction via TGF-β1 and whether it is through the same induction pathway as collagen contraction-induced apoptosis.

Chapter 6

Is TGF-β1 Involved in Collagen Contraction-Induced Apoptosis?

6.1 Introduction

Results from Chapters 3 and 4 provide evidence that the apoptosis which is induced during collagen contraction is most likely through the transduction of mechanical signals into biochemical signals, apparently involving MMP activation. It is possible that MMPs drive apoptosis through the degradation of the extracellular matrix and production of specific small soluble extracellular matrix fragments that are capable of inducing apoptosis. Furthermore, Chapter 5 demonstrates that TGF-β1 can also induce apoptosis specifically in 3-D collagen and thus may also be implicated in this apoptosis signalling cascade.

It is possible that the effects of TGF-β1 are simply through induction of myofibroblast differentiation. Myofibroblasts may be the cell phenotype capable of responding to apoptosis cues or further still, may be primarily responsible for producing the apoptosis cues. Results from Chapter 5, show that cell death does appear to correlate with the presence of myofibroblasts, which form in normal scar cell populated gels during the period the collagen matrix is retained anchored. Results from keloid scar fibroblasts presented in Chapter 5 further demonstrates, that their inability to undergo collagen contraction-induced apoptosis coincides with the absence of myofibroblast formation in collagen gels. Furthermore, with the addition of exogenous active TGF-β1, myofibroblast differentiation does take place in keloid scar-seeded matrices, and so does apoptosis.

It is not understood why the addition of exogenous active TGF-β1 induces apoptosis in anchored collagen matrices; perhaps TGF-β1 leads to the removal of cell tension and contacts in a similar manner to that seen during the relaxation (contraction) of the attached-stressed collagen gels, but through another mechanism. In normal scar cell-seeded collagen matrices maintained in minimal growth media alone, myofibroblast differentiation occurs when matrices are anchored, yet apoptosis only occurs when the collagen matrices contract. This suggests that the presence of myofibroblasts alone in collagen gels is not sufficient to cause apoptosis. Furthermore, in consideration of the fact that even in the absence of TGF-β1 treatment nearly all the normal scar fibroblasts present in collagen gels have differentiated into myofibroblasts, the TGF-β1 treatment induction of apoptosis is unlikely to be simply due to an increase in

myofibroblast proportion as previously hypothesised. It is feasible that either the addition of exogenous active TGF-β1 or collagen contraction causes some sort of maturation of the myofibroblast that we have not been able to detect.

This therefore leaves the question of whether TGF-β1's role during wound healing is to simply induce myofibroblast differentiation, or whether there is a further separate role for TGF-β1 specific to collagen contraction, which leads to apoptosis. The continued presence of TGF-β1 in the wound site throughout the wound healing process, during wound resolution, (Frank *et al.,* 1996) may point to a previously overlooked aspect of TGF-β1's many pleiotropic effects. In addition, if TGF-β1 does have a separate role in collagen contraction-induced apoptosis other than myofibroblast differentiation, this may be the controlling factor preventing cellular apoptosis within fibrin matrices.

6.1.1 TGF-β1 is a Multifunctional Cytokine

TGF-β1 is one of three TGF-β isoforms that are members of a family of signalling molecules (Massague, 1998). TGF-β1 has been intensely studied in relation to its effects on wound repair and its role in fibrotic pathologies (Martin *et al.,* 1992; Roberts and Sporn, 1993; Blobe *et al.*, 2000). The main role of TGF-β1 during wound healing is thought to be as a potent activator of fibroblasts, inducing their differentiation into myofibroblasts and stimulating their production of extracellular matrix (Grotendorst *et al.*, 1997; Leask *et al.*, 2004).

TGF-β1 also has the capacity to modulate many other processes; including early development, differentiation, extracellular matrix formation, haematopoiesis, angiogenesis, immune functions, and apoptosis induction (Schuster and Krieglstein, 2002). TGF-β1 has both stimulatory and inhibitory effects depending on its concentration, the cell type its acting on, the cell density and cellular microenvironment (Grande, 1997). Furthermore, TGF-β1 possesses both pro- and anti-inflammatory activities as dramatically illustrated in the TGF-β1 knock-out mouse model, in which the animals died with systemic inflammation (Crowe *et al.*, 2000).

6.1.2 About the TGF-β1 Molecule

TGF-β1 is secreted as an inactive, high molecular weight precursor complex (Lawrence *et al.*, 2001; Wakefield *et al.*, 1988; Miyazono *et al.*, 1988; Harpel *et al.*, 1992). The receptors for TGF-β1 are almost universally expressed by cells (Wakefield *et al.*, 1987); leading to the suggestion that local activation of latent TGF-β1 may be important in regulating cellular responses to this molecule. The TGF-β1 gene encodes a 390-amino acid precursor molecule, which contains a signal peptide, the active TGF-β1 molecule, and the latency associated peptide (LAP) (Gentry *et al.*, 1988), see Figure 6.1.

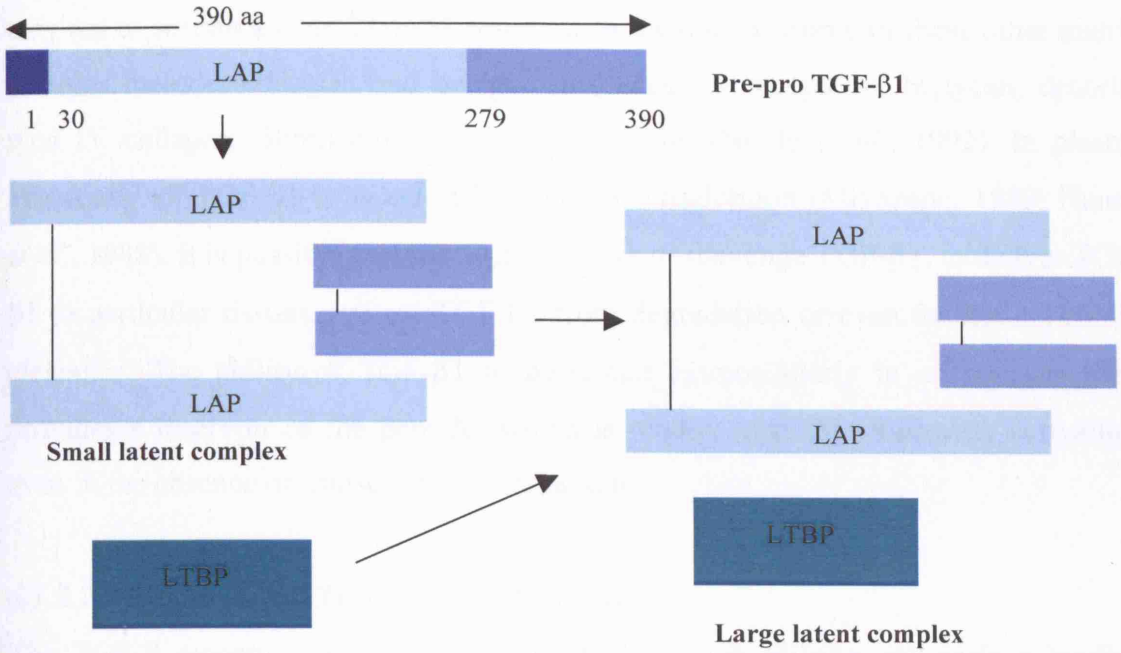


Figure 6.1 Latent TGF-β1 complexes. Following removal of the signal peptide (amino acid (aa) residues 1-29), the gene product undergoes proteolytic cleavage between two arginine residues at positions 278 and 279 to produce mature TGF-β1 (residues 279-390, and LAP residues 30-278). Prior to secretion, disulphide-linked homodimers of TGF-β1 noncovalently associate with homodimers of LAP to produce the small latent TGF-β1 complex, which are inactive. In some cells, the small latent complex associates with an additional 125-160 kDa protein, latent TGF-β-binding protein (LTBP) to produce the large latent complex (adapted from Grande, 1997).

Latent TGF-β1 produced by fibroblasts may be activated by proteases such as plasmin, cathepsin D (Lyons *et al.*, 1988), elastase and even MMPs (Dallas *et al.*, 2002). Carbohydrate structures may also play a role in TGF-β activation. Latent TGF-

β1 contains mannose-6-phosphate residues (Roberts and Sporn, 1996) and can bind to mannose-6-phosphate (M-6-P) receptors for activation (Kovacina *et al.*, 1989). Glycosidase, sialic acid and sialidase treatment also activate TGF-β1 (Miyazono, 1989). Once activated, TGF-β1 binds other serum and matrix proteins as described in the section below.

6.1.2.1 Location of TGF-β1 Molecules in the Extracellular Matrix Environment

Diverse proteins bind TGF-β1, of which the cell membrane proteins TGF-β receptors I and II are most well known (Massague, 1992). Other TGF-β binding proteins do not appear to participate in TGF-β signal transduction like receptors I and II; but instead may act to sustain a local TGF-β1 reservoir in the matrix. Some of these other matrix proteins include: endoglin and betaglycan (accessory receptors), biglycan, decorin, type IV collagen, fibronectin and thrombospondin (Noble *et al.*, 1992). In plasma essentially all TGF-β1 is associated with α_2-macroglobulin (Miyazono, 1989; Huang *et al.*, 1988). It is possible that these proteins act to scavenge TGF-β1, to deliver TGF-β1 to particular tissues, protect TGF-β1 from degradation or even facilitate TGF-β1 clearance. The ability of TGF-β1 to be stored extracellularly in an inactive form provides a reservoir of the peptide, which is readily available following activation, even in the absence of transcriptional induction.

6.1.2.2 TGF-β1 Signal Transduction Pathway

The TGF-β receptor types I and II are distinct high affinity cell-surface binding receptors that are required for signal transduction (Wrana *et al.*, 1992; Attisano *et al.*, 1994). These receptors are unique in that they function as membrane-bound serine/threonine protein kinases (Massague, 1992). TGF-β signalling is thought to begin with ligand binding to the type II receptor, this then associates with and phosphorylates the cytoplasmic domain of the type I receptor (Wrana *et al.*, 1994) (see Figure 6.2). Phosphorylation of type I receptor by type II receptor is necessary for signalling (Carcamo *et al.*, 1995). The activated type I receptor then phosphorylates cytoplasmic proteins, which transduce the signal into the nucleus (Boyd and Massague, 1989). Betaglycan (TGF-β receptor III) can also bind TGF-β1 and acts to present the cytokine to the kinase subunit of the signalling receptor, enhancing cellular responses to TGF-β (Lopez-Casillas *et al.*, 1994).

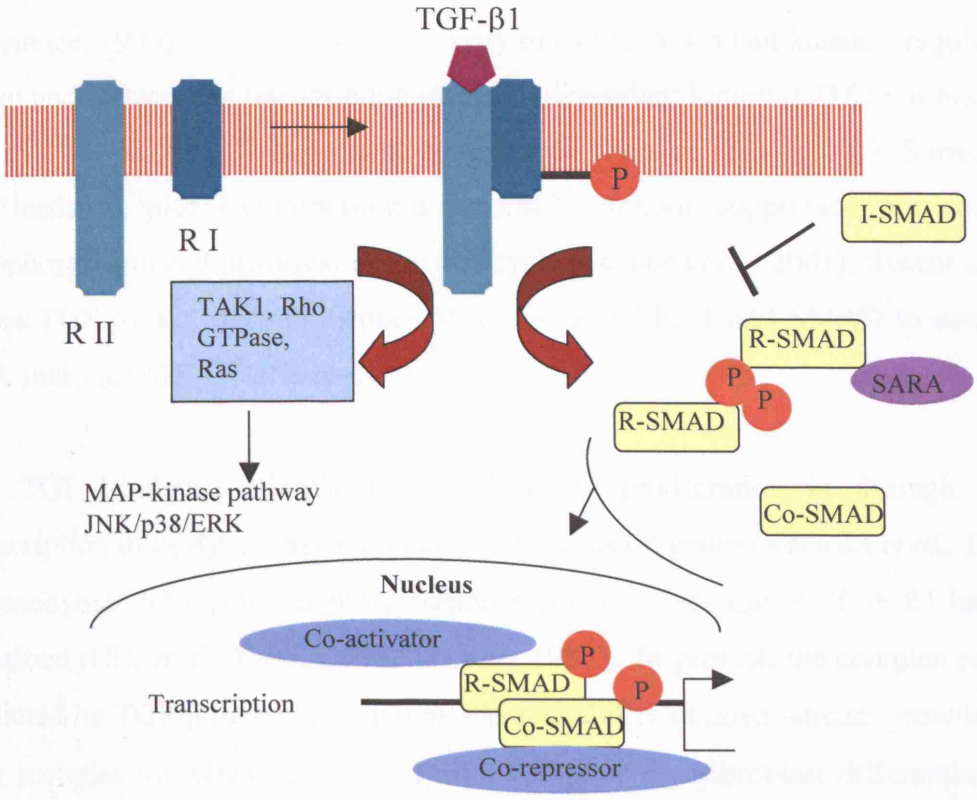


Figure 6.2 Schematic representation of the TGF-β1 signal transduction pathway. Ligand binding to TGF-β receptors leads to the formation of heterotetrameric complexes of receptors I and II. TGF-βRII phosphorylates and activates TGF-βRI. This leads to the internal phosphorylation of receptor activated SMADs (SMAD 2/3), which are released from a complex with SARA (SMAD anchor for receptor activation). These R-SMADs form a complex with a co-SMAD (SMAD-4) which leads to translocation into the nucleus, to interact with other co-activators or co-repressors for gene transcription. This pathway can be inhibited at the receptor level by inhibitory SMADs (SMAD-7), or after activation R-SMADs are targeted for degradation via the ubiquitin-proteasome pathway. TGF-β1 also mediates the TAK1 (TGF-β-activated kinase 1) pathway, Rho GTPase and Ras pathways. TAK1 mediates TGF-β1 effects with respect to the activation of distinct MAP kinase pathways (JNK and p38 stress activated protein kinase). Rho GTPase and Ras mediate TGF-β1 effects through ERK signalling.

6.1.3 Proliferative and Apoptotic Effects of TGF-β1 on Specific Cell Types

Research to date on the effects of TGF-β1 has generally found that TGF-β1 inhibits cell proliferation of epithelial cells (Shipley *et al.,* 1986; Tucker *et al.,* 1984) and endothelial cells (Takehara *et al.,* 1987; Baird and Durkin, 1986), whereas it stimulates the proliferation of fibroblasts (Sporn and Roberts, 1992; Gold, 1999). Inhibition of growth by TGF-β1 is through blockage of the cell cycle in late G1 phase,

and has been linked to its ability to down-regulate the proto-oncogene c-Myc (Lawrence, 1996). This inhibits the activity of cyclin dependant kinases (regulates cell cycle) and induces the transcription of cyclin dependant kinase (CDK) inhibitors such as $p15^{INK4b}$, $p21^{Cip1/Waf1}$ dependant on cell type (Seoane *et al.*, 2001). Subsequently this leads to pRb (retinoblastoma protein – tumour suppressor protein) hypo-phosphorylation and the arrest of the cell cycle (Seoane *et al.*, 2001). Recent evidence shows TGF-β1 not only influences Myc, but also Miz-1 and SMAD to control the CDK inhibitor $p15^{INK4b}$ (Seoane *et al.*, 2001).

The TGF-β-induced stimulation of fibroblast proliferation is through positive transcription of c-Myc, v-Sis, collagen and fibronectin genes (Kataoka *et al.*, 1993). In mesenchymal cells however more complex biphasic responses to TGF-β1 have been described (Hill *et al.*, 1986; Centrella *et al.*, 1987). In general, the complex responses mediated by TGF-β1 are controlled by the availability of down-stream growth factors. This includes for example, TGF-β1 stimulation of myofibroblast differentiation and stimulation of extracellular matrix production is through CTGF signalling (Grotendorst *et al.*, 2004), and TGF-β1 stimulation of fibroblast proliferation is through PDGF signalling (Lawrence, 1996).

The effects of TGF-β1 on growth arrest and apoptosis has been studied by numerous groups (Wahl *et al.*, 1988; Chaouchi *et al.*, 1995; Lomo *et al.*, 1995). It has been discovered that the growth inhibitory/proliferative effects of TGF-β1 are separate from TGF-β1's apoptotic effect (Schuster and Krieglstein, 2002). The induction of apoptosis by TGF-β1 has been investigated in a large variety of cell types including lymphoma, hepatoma, endothelial, epithelial and transformed fibroblasts from different animal models, reviewed by Jarpe *et al.* (1998) and Schuster and Krieglstein, (2002). They summarised that the main effector pathways of apoptosis utilised by TGF-β1 involved: cooperation with Fas, TNF-α, nerve growth factor (NGF), activation of the JNK cascade, SMAD activation, cooperation with AP-1 transcription factor, activation of TGF-β-inducible early gene (TIEG), generation of reactive oxygen species (ROS), down-regulation of $Bcl\text{-}X_L$/Bcl-2, cytochrome c release, release of apoptosis-related protein in TGF-β signalling pathway (ARTS) from the mitochondria, activation of calpain and caspases.

No research has been published to date specifically linking TGF-β1 with the apoptosis of normal human fibroblasts or indeed in the clearance of wound cells. In general TGF-β1 has been found to inhibit fibroblast/myofibroblast apoptosis (Zhang and Phan, 1999; Chen *et al.*, 2003a). These *in vitro* experiments however, studied the effects of TGF-β1 in monolayer culture. As described previously in Chapter 3, monolayer culture does not effectively mimic the *in vivo* situation. The culture of fibroblasts in 3-D collagen gels has been used as a model that more closely resembles wound tissue (Grinnell, 1994). It is possible that in view of the many pleiotropic effects of TGF-β1, this cytokine may affect fibroblasts differently in a 3-D collagen based environment than that reported in monolayer culture. Thus, in consideration of the phenomenon that TGF-β1 induces apoptosis of fibroblasts, previously presented in Chapter 5, TGF-β1 may have a role in the cell clearance that occurs at the end phase of wound healing.

6.1.4 TGF-β1 Secretion Patterns During Wound Healing

Studies into the endogenous secretion patterns of TGF-β1 upon injury describe that at the time of injury the initial source of TGF-β1 is through the release from its latent form from degranulating platelets into the wound bed. The released TGF-β1 signals chemotaxis and activation of other inflammatory and wound cells, leading to further increases in endogenous TGF-β1 levels via a positive feedback loop (Frank *et al.*, 1996). It is thought that at this point TGF-β1's effect on fibroblasts within the provisional fibrin matrix is to encourage cell migration, proliferation and differentiation, as detailed previously in Chapter 1. This role for TGF-β1 in the early wound environment is corroborated by the results in Chapter 5; where cell proliferation is detected in normal scar fibroblast-seeded fibrin matrices in the presence of TGF-β1, mimicking the early wound environment. However, TGF-β1 is present at high levels for the duration of wound healing, well after the fibro-proliferative stage is complete, a finding that is suggestive of a further potential role for this cytokine in the later stages of wound healing (Frank *et al.*, 1996).

6.1.5 Aim

This chapter aims to determine if TGF-β1 does indeed take part in the apoptosis that is induced during collagen contraction. Further, it aims to distinguish the exact nature of this role determining if it is simply via:

- Signalling the differentiation of a cell type that is susceptible to these apoptosis cues.
- Its effects on collagen breakdown.
- Or an alternative distinct mechanism whereby TGF-β1 acts as a permissive co-factor that is required for apoptosis.

6.2 Results

Note that some of the following results are from multiple large complex experiments designed to address several hypothesis. Parts of these results have therefore been presented earlier for clarity and are marked as such in text, but are included as necessary comparisons later in the text where the whole experiment is presented.

6.2.1 Does TGF-β1 have a Specific Role in Collagen Contraction-Induced Apoptosis or are its Effects through Myofibroblast Differentiation Alone?

In order to determine if the identified novel role of TGF-β1 in inducing apoptosis in a 3-D collagen environment plays a role in collagen contraction-induced apoptosis, agents capable of blocking the activity of TGF-β1 were added to the *in vitro* wound healing model. Specifically, M-6-P was used to block the activation of autocrine latent-TGF-β1, which requires binding to the M-6-P receptor for its activation (Kovacina *et al.*, 1989). Also, an antibody known to bind TGF-β1 (anti-TGF-β1) and block its activity was used. In addition, to gain an indication as to whether the ability of TGF-β1 to induce cell death within collagen gels is simply through myofibroblast differentiation, or whether TGF-β1 has an additional but separate role in the apoptosis that is induced during collagen contraction; these blocking agents were added at different time-points. The TGF-β1 blocking agents were either added at the beginning of the collagen gel culture period (day-0) in order to prevent myofibroblast

differentiation or later, on the release of contractile collagen matrices (day-4); in this manner allowing myofibroblast differentiation to take place during the period the collagen gel is maintained anchored. The latter will determine whether the presence of TGF-β1 is needed alongside the presence of myofibroblasts in order for successful apoptosis induction during collagen contraction. For this investigation experiments were performed on normal scar-derived fibroblasts only as they are considered a 'normal' phenotype.

Figure 6.3 and 6.4 demonstrate the effect of M-6-P and anti-TGF-β1 on normal scar cells in contractile and anchored collagen gels. When these agents were added at day-0 of collagen matrix culture, as predicted, fibroblasts failed to differentiate into myofibroblasts (Figure 6.3B). In addition the significant (p=<0.001) induction of cell death seen in contractile collagen matrices maintained in minimal growth medium alone was completely inhibited (Figure 6.3A).

Mannose-6-Phosphate or Anti-TGF-β1 added at Day-0

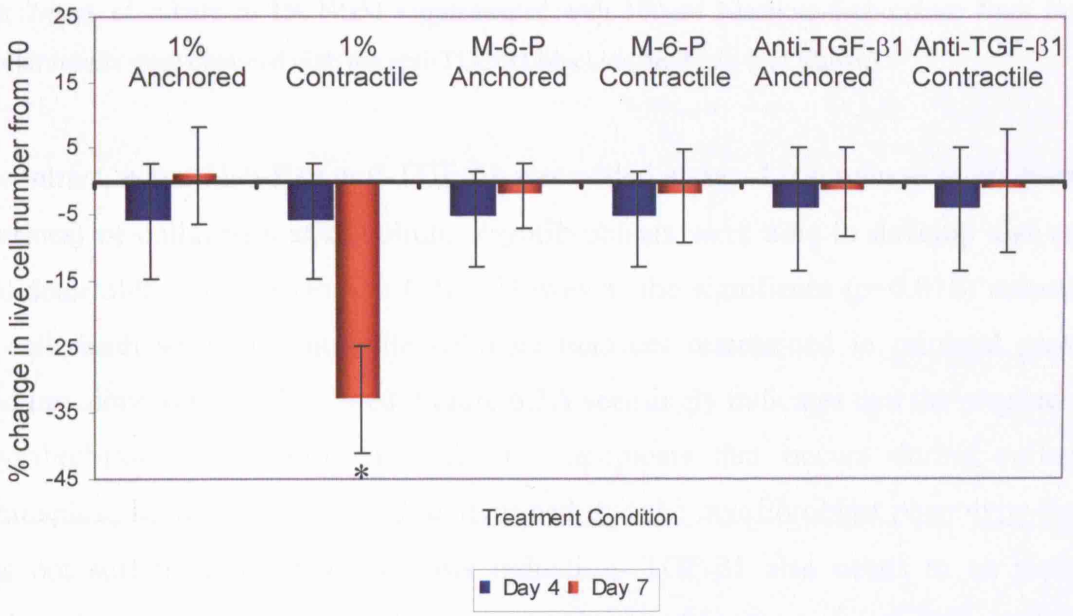


Figure 6.3A Collagen contraction-induced apoptosis with or without the addition of anti-TGF-β1 (2ng/ml) or M-6-P (100μM) to the 1% NGM at day-0 (24hrs after seeding gels) of matrix culture. This experiment was carried out with normal scar cells. Viable cell number was assessed at day-0, -4 and -7 by Trypan Blue exclusion. The results represent the experiment carried out in triplicate with n=5 normal scar cell strains. Error bars represent SD. T-test analysis compared the percentage change in live cell number in contractile vs anchored gels between each treatment condition at day-4 and day-7. *P =<0.05.

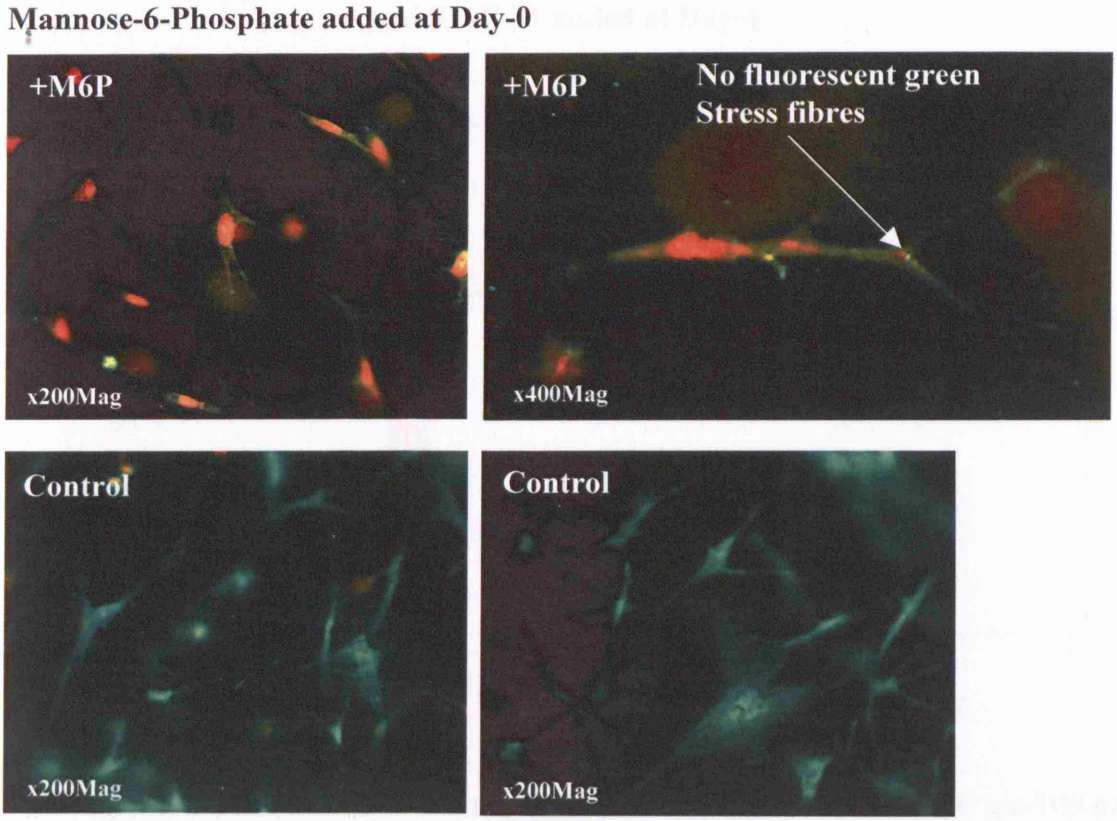


Figure 6.3B Typical α-SMA staining of normal scar-derived fibroblasts in anchored collagen matrices after 7-days of culture in 1% NGM supplemented with 100μM Mannose-6-phosphate from day-0. Similar results were obtained with the anti-TGF-β1 blocking antibody (not shown).

In contrast, when M-6-P or anti-TGF-β1 was added at day-4 (on release of contractile matrices) of collagen matrix culture, myofibroblasts were able to develop and were still detectable at day-7 (Figure 6.4B). However, the significant ($p=0.018$) induction of cell death seen in contractile collagen matrices maintained in minimal growth medium alone was still inhibited. Figure 6.3A seemingly indicates that the presence of myofibroblasts is a requirement for the apoptosis that occurs during collagen contraction; however Figure 6.4A determined that the myofibroblast phenotype alone was not sufficient for this apoptosis induction. TGF-β1 also needs to be present during the contraction of collagen matrices, in this manner possibly inducing apoptosis through a separate signalling pathway to that of myofibroblast differentiation.

Mannose-6-Phosphate or Anti-TGF-β1 added at Day-4

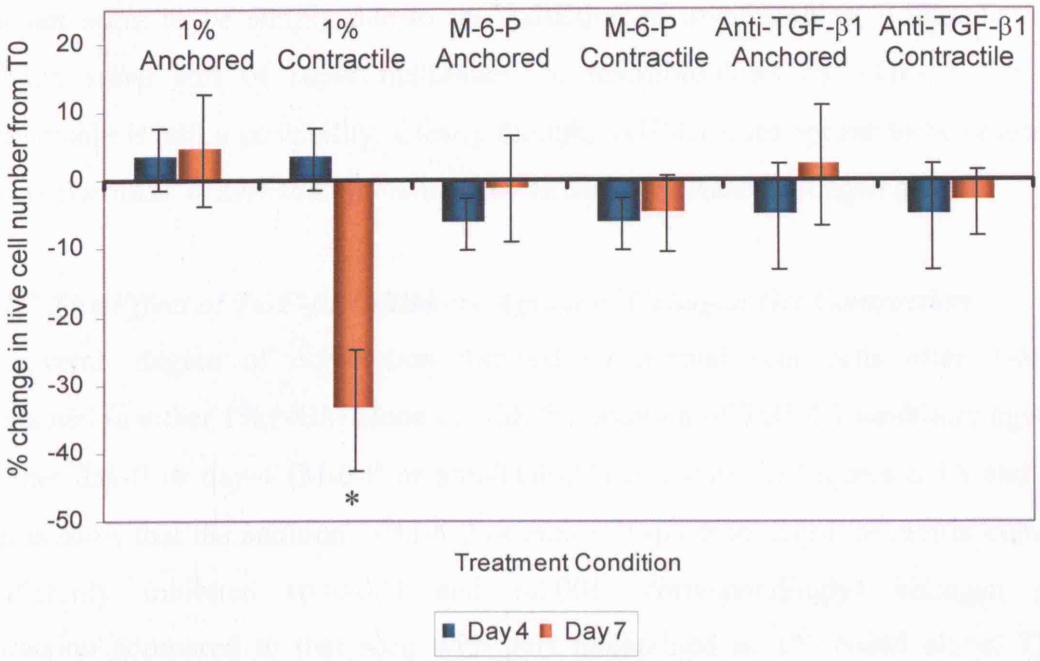


Figure 6.4A Collagen contraction-induced apoptosis with or without the addition of anti-TGF-β1 (2ng/ml) or mannose-6-phosphate (100μM) to the 1% NGM at day-4 of matrix culture. These experiments were carried out with normal scar cells. Viable cell number was assessed at day-0 (24hrs after seeding gels), 4 and 7 by Trypan Blue exclusion. The results represent the experiment carried out in triplicate with n=5 normal scar cell strains. Error bars represent SD. T-test analysis compared the percentage change in live cell number in contractile vs anchored gels between each treatment condition at day-4 and day-7. *P =<0.05.

Mannose-6-Phosphate added at Day-4

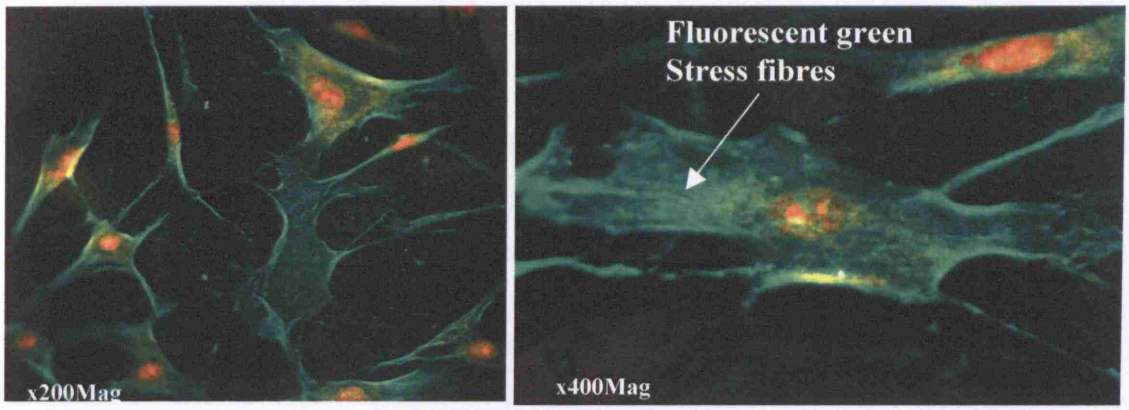


Figure 6.4B Typical α-SMA staining of normal scar-derived fibroblasts in anchored collagen matrices after 7-days of matrix culture in 1% NGM supplemented with 100μM Mannose-6-phosphate from day 4 onwards. Similar results were obtained with the anti-TGF-β1 blocking antibody (not shown).

These results show that the activity of TGF-β1 is an absolute requirement for the apoptosis induced during collagen contraction. Furthermore, the action of TGF-β1 does not seem to be simply due to the induction of myofibroblast differentiation, although some sort of super maturation of myofibroblasts by TGF-β1 that is undetectable is still a possibility. Clearly though, TGF-β1 does appear to be required for myofibroblast differentiation induced by tension in stressed collagen gels.

6.2.1.1 The Effect of TGF-β1 Inhibitory Agents of Collagen Gel Contraction

The overall degree of contraction induced by normal scar cells after 7-days maintained in either 1% NGM alone or with the addition of TGF-β1 inhibitory agents at either day-0 or day-4 (M-6-P or anti-TGF-β1) is shown in Figures 6.5A and B. Results show that the addition of M-6-P or anti-TGF-β1 from day-0 of matrix culture significantly inhibited ($p=0.003$ and <0.001, correspondingly) collagen gel contraction compared to that seen with gels maintained in 1% NGM alone. This correlates with results presented in Figure 6.3B, where no myofibroblasts are present in M-6-P or anti-TGF-β1 treated gels. When M-6-P or anti-TGF-β1 were added at day-4 however, there was no significant difference between the degree of contraction induced by cells maintained in 1% NGM alone or with the addition of M-6-P or anti-TGF-β1. Normal scar cells contracted each of these matrices by ~50% after 7-days of collagen matrix culture, irrespective of the culture medium (Figure 6.5A and B). This was not surprising as Figure 6.4B shows that there is no difference in myofibroblast differentiation at day-7 of gel culture. These results further confirm that myofibroblasts themselves along with contraction is insufficient to lead to apoptosis.

Figure 6.5A and B also shows that the degree of contraction is not relative to the amount of apoptosis taking place within the matrix (Figure 6.3A and 6.4A). This corroborates results presented in Chapter 3 comparing contraction induced by keloid scar and normal scar cells, where the degree of contraction did not correlate with whether apoptosis took place within the matrix.

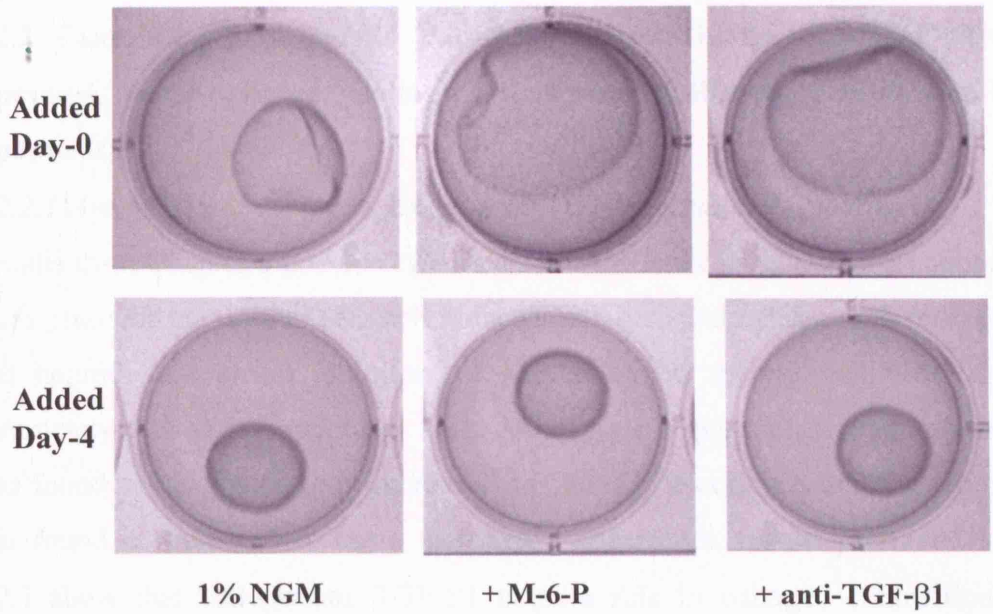


Figure 6.5A Typical contraction of collagen matrices seeded with normal scar fibroblasts at day-7 of matrix culture. Cells were maintained in 1% NGM supplemented with or without Mannose-6-Phosphate (100μM) or anti-TGF-β1(2ng/ml) added at day-0 (24hrs after seeding gels) or day-4 of matrix culture.

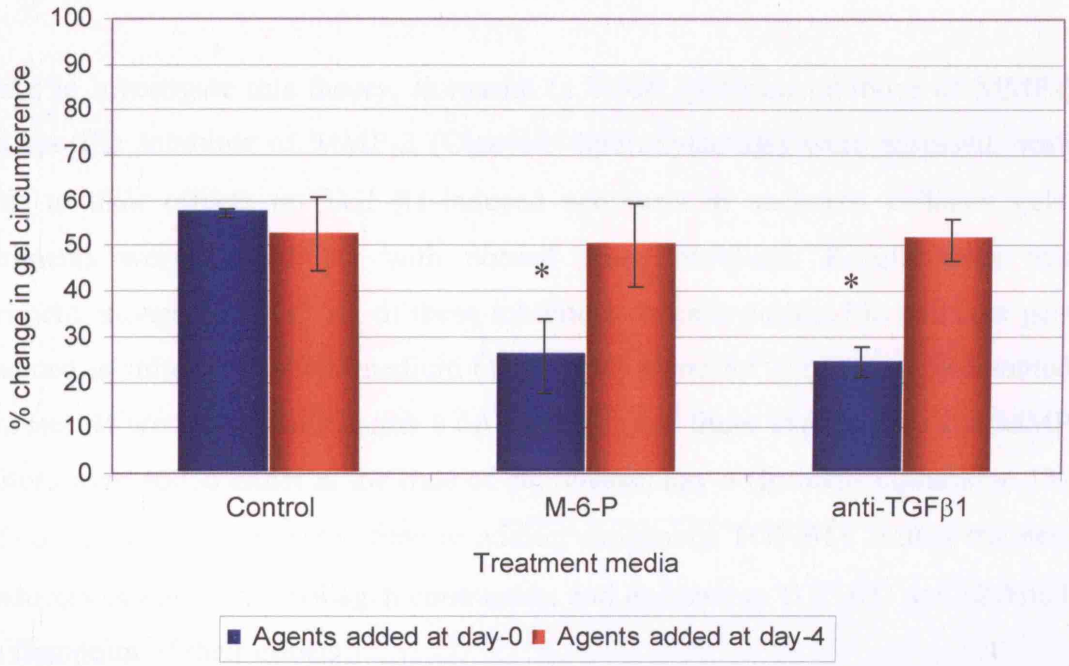


Figure 6.5B Graph to show the mean percentage change in gel circumference to that of the perimeter of the tissue culture well (original circumference of the gel). The degree of contraction was measured at day-7 of collagen matrix culture. Normal scar cells were maintained in 1% NGM supplemented with or without Mannose-6-Phosphate (100μM) or anti-TGF-β1(2ng/ml) added at day-0 (24hrs after seeding gels) or day-4 of matrix culture. T-test analysis compared the percentage change in gel circumference after M-6-P or anti-TGF-β1 treatment to that of control treated gels. *P=<0.05.

6.2.2 Examining Mechanistic Parallels between Exogenous TGF-β1-Induced Apoptosis in Anchored Collagen Gels and Collagen Contraction-Induced Apoptosis

6.2.2.1 Are MMPs Involved in Exogenous TGF-β1-Induced Apoptosis?

Results from Chapter 4 provide evidence that MMPs are involved in the apoptosis that takes place during normal scar cell induced collagen contraction; with pharmaceutical and naturally occurring inhibitors of MMPs and a specific inhibitor of MMP-2 completely preventing apoptosis. Both MMP gene expression and protein activation was found to be significantly increased in contractile collagen matrices compared to that found in anchored collagen matrices. Furthermore, results presented in section 6.2.1 show that endogenous TGF-β1 plays a role in collagen contraction-induced apoptosis, separate from that of detectable myofibroblast differentiation but specific to that of collagen contraction. It is known that TGF-β1 can affect MMP gene expression and protein activation (Roberts *et al.*, 1992). It is therefore possible that exogenous TGF-β1 induction of apoptosis in anchored collagen gels is simply due to induction of MMP activity and thus matrix breakdown.

In order to investigate this theory, Ilomastat (a broad spectrum inhibitor of MMPs) and a specific inhibitor of MMP-2 (Oleoyl-N-hydroxylamide) were assessed, with regards to their effects on TGF-β1-induced apoptosis in anchored collagen gels. Experiments were carried out with normal scar fibroblasts. Results from this experiment, alongside the effect of these inhibitors on cells cultured in collagen gels maintained in minimal growth medium (1% NGM) alone for comparison (presented in Chapter 4) are shown in Figures 6.6A and 6.7. For these experiments the MMP inhibitors were added either at the time of gel release, day-4 (for cells cultured in 1% NGM) or at day-0 (at the same time as adding exogenous TGF-β1). In this manner, the inducers of apoptosis (collagen contraction and exogenous TGF-β1) are inhibited at the first point of their effect.

As previously discussed in Chapter 4, the broad spectrum inhibitor Ilomastat is capable of inhibiting the significant ($p=0.024$) induction of apoptosis that occurs during collagen contraction in minimal growth medium alone. However, Ilomastat appears to have no such effect on the apoptosis induced in the presence of TGF-β1 at

either day-4 or day-7. Figure 6.6A shows that the addition of Ilomastat to these gels actually increases apoptosis by ~10% at day-4 (not significantly) and by ~20% by day-7 (p=0.049) (Table 6.1).

This apparent lack of affect could be due to Ilomastat being toxic, thereby masking any inhibitory affect on apoptosis due to blocking MMP activity by inducing death in its own right. However, titration of the toxic effect of Ilomastat performed over a 7-day period on normal scar cells cultured in anchored collagen gels, showed that no toxicity was observed by Ilomastat at the concentration used (Figure 6.6B).

Similar to the results with Ilomastat, the specific inhibitor of MMP-2 can inhibit the apoptosis induced during collagen contraction (Figure 6.7 and as previously presented in Chapter 4). However, unlike Ilomastat, specifically inhibiting MMP-2 does appear to reduce the apoptosis induced in collagen gels in the presence of TGF-β1 at 4-days but not after 7-days. On comparing the degree of apoptosis induced by exogenous TGF-β1 at day-4, the MMP-2 inhibitor significantly (p=<0.001) reduced the induction of apoptosis by ~30%. Nevertheless, apoptosis was still induced to a significant degree by TGF-β1 in the presence of this inhibitor (p=0.036) compared to cells maintained in anchored gels in minimal growth medium alone (Table 6.1). By day-7, the degree of apoptosis detected in collagen gels in the presence of TGF-β1 and the MMP-2 inhibitor was comparable to that seen in TGF-β1-treated matrices in the absence of the MMP-2 inhibitor (Figure 6.7). These results indicate that the apoptosis induced by the exogenous addition of TGF-β1 can be reduced in the early stages by specifically inhibiting MMP-2, but not after 7-days. These results appear to contradict the findings with the broad spectrum MMP inhibitor Ilomastat, but may indicate that MMP-2 is specifically involved in TGF-β1-induced apoptosis in collagen gels. The apparent contradiction might be explained by differences in the strength of MMP-2 inhibition by the two antagonists.

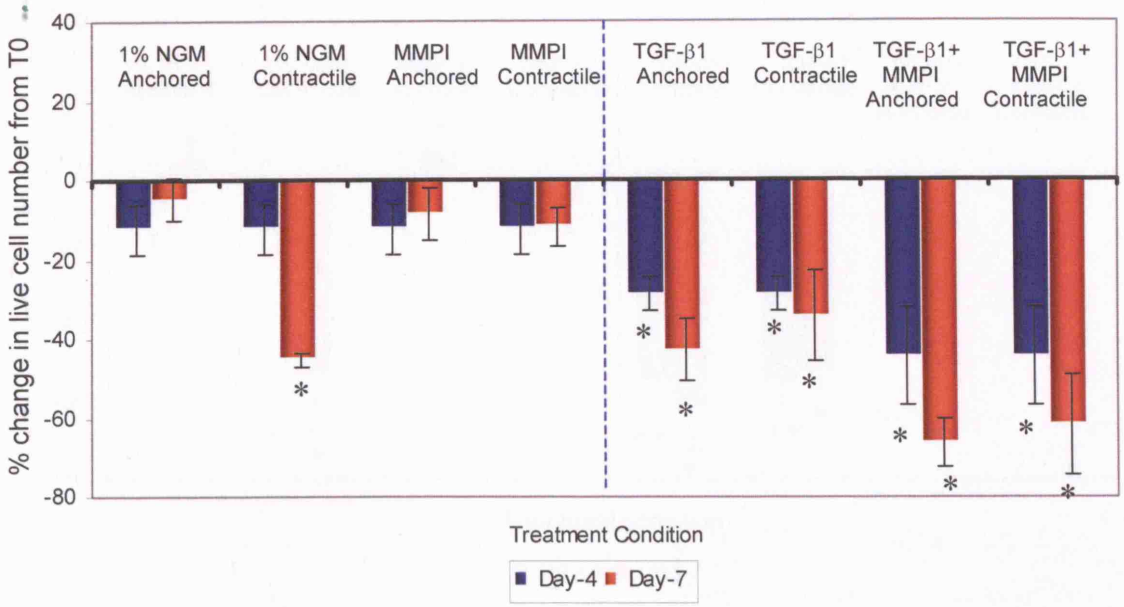


Figure 6.6A Collagen contraction-induced apoptosis with or without the addition of Ilomastat at 5μM (GM6001). Ilomastat (MMPI) was added to the 1% NGM at day-4, on releasing contractile matrices or day-0 (24hrs after seeding the gels), on adding exogenous TGF-β1. Normal scar-derived fibroblasts were seeded into collagen matrices and viable cell number was assessed at day-0, -4 and -7 by Trypan Blue exclusion. This graph shows the percentage change in live cells at day-4 and -7 from day-0. These results represent the experiment carried out in triplicate with n=4 normal scar cell strains. Error bars represent SD. T-test analysis compared the effect of different treatment conditions on live cell number in anchored and contractile gels at day-4 and day-7 vs 1% NGM alone. *P = <0.05.

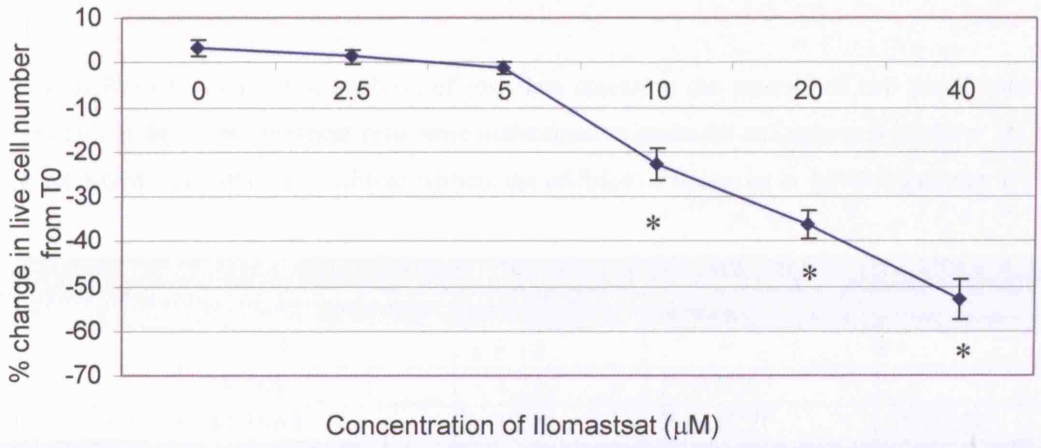


Figure 6.6B Ilomastat titration assay with normal scar fibroblasts. Cells were cultured in anchored collagen gels maintained in 1% NGM with 0, 2.5, 5, 10, 20 or 40μM of Ilomastat added to the culture media. Cells were treated for 7-days before assessing viable cell number by Trypan Blue exclusion. This graph shows the % change in live cells at day-7 from day-0 (before adding treatment media). These results represent the experiment carried out in triplicate with n=3 normal scar cell strains. Error bars represent SD. *P = <0.05 by T-test analysis.

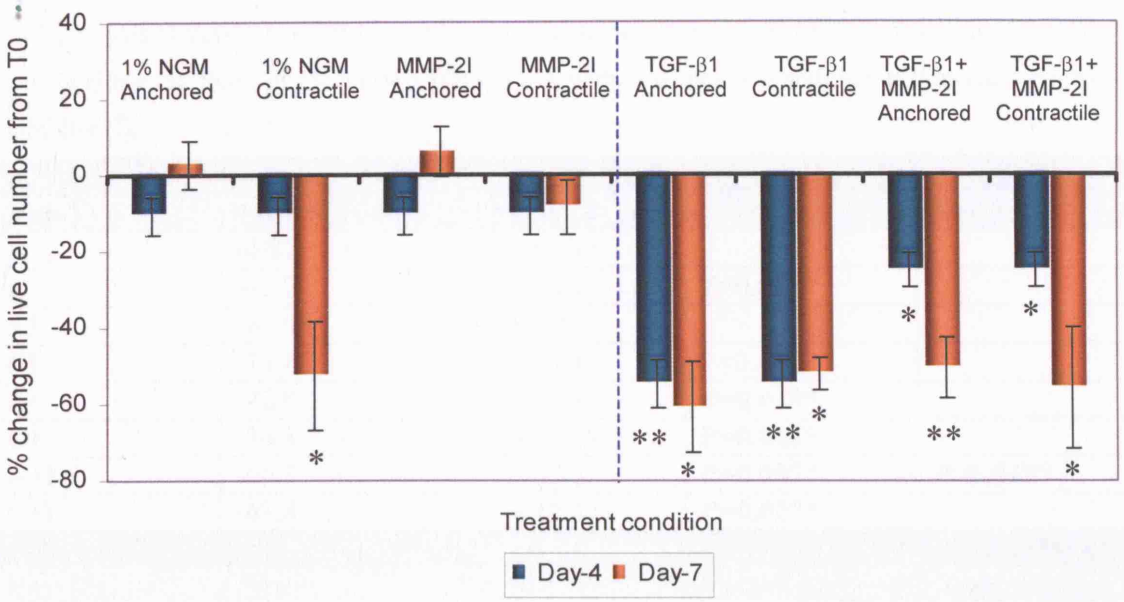


Figure 6.7 Collagen contraction-induced apoptosis with or without the addition of a specific MMP-2 Inhibitor at 10μM. The MMP-2 inhibitor (MMP-2I) was added to the 1% NGM at day-4, on releasing contractile matrices or day-0 (24hrs after seeding the gels), on adding exogenous TGF-β1. Normal scar-derived fibroblasts were seeded into collagen matrices and viable cell number was assessed at day-0, -4 and -7. This graph shows the percentage change in live cells at day-4 and -7 from day-0. These results represent the experiment carried out in triplicate with n=4 normal scar cell strains. Error bars represent SD. T-test analysis compared the effect of different treatment conditions on live cell number in anchored and contractile gels at day-4 and day-7 vs 1% NGM alone. **P = <0.001 and *P = <0.05.

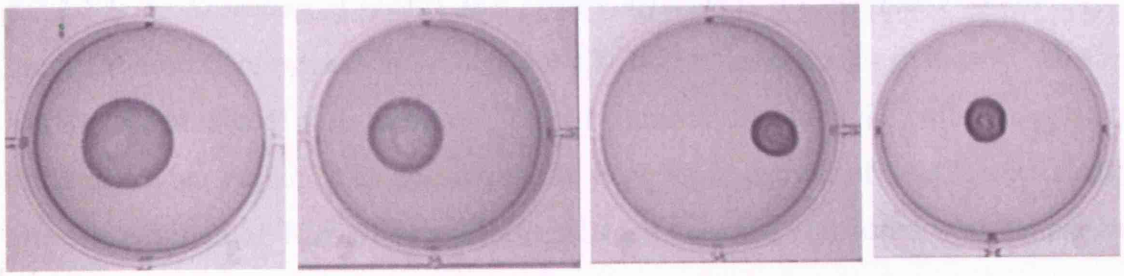
Table 6.1A Results from T-test analysis of raw data assessing the amount of cell death induced in collagen gels at day-4. Normal scar cells were maintained in anchored collagen gels in either 1% NGM (A) or 1% NGM +TGF-β1 (A+), with or without the addition of Ilomastat or MMP-2 inhibitor (I).

Ilomastat				
Day-4	**Mean % death**	**± SD**	**Versus A**	**Versus A+**
A	-12.378	± 6.18		
A+	-28.609	± 4.25	P=0.026*	
A+I	-44.3963	± 12.51	P=0.027*	P=0.136
MMP-2 Inhibitor				
Day-4	**Mean**	**SD**	**Versus A**	**Versus A+**
A	-10.66	± 4.94		
A+	-54.88	± 6.25	P=<0.001**	
A+I	-25.13	± 4.49	P=0.036*	P=<0.001**

Table 6.1B Results from T-test analysis of raw data assessing the amount of cell death induced in collagen gels at day-7. Normal scar cells were maintained in anchored (A) or contractile (C) collagen gels in either 1% NGM or 1% NGM +TGF-β1 (+), with or without the addition of Ilomastat or MMP-2 inhibitor (I).

Ilomastat				
Day-7	**Mean % death**	**± SD**	**Versus A**	**Versus A+**
A	-4.871	± 5.37		
C	-45.24	± 1.89	**P=0.024***	
AI	-8.646	± 6.46		
CI	-11.88	± 4.64	P=0.543	
A+	-42.9	± 7.75	**P=0.039***	
C+	-34.3	± 11.27	**P=0.042***	
A+I	-66.3	± 6.26	**P=0.007***	**P=0.049***
C+I	-61.8	± 12.47	**P=0.027***	
MMP-2 Inhibitor				
Day-7	**Mean**	**SD**	**Versus A**	**Versus A+**
A	2.53	± 6.44		
C	-52.54	± 14.35	**P=0.004***	
AI	6.01	± 6.59		
CI	-8.50	± 7.09	P=0.108	
A+	-60.82	± 11.64	**P=0.002***	
C+	-52.04	± 4.22	**P=<0.001****	
A+I	-50.44	± 7.89	**P=<0.001****	P=0.234
C+I	-55.81	± 15.86	**P=0.003***	

The overall degree of contraction induced by normal scar fibroblasts was assessed with cells maintained in either 1% NGM or 1% NGM + TGF-β1 in the presence or absence of Ilomastat (Figure 6.8A and B). As previously found, a significant increase ($p=0.041$) in matrix contraction was detected in gels that had been maintained in the presence of TGF-β1 compared to gels maintained in 1% NGM alone. Ilomastat however, appeared to have no significant effect on matrix contraction, even in the presence of TGF-β1 (Figure 6.8B). Clearly, these results corroborate those found earlier in Chapters 3 and 4, that contraction is not necessarily followed by apoptosis. In addition, MMP activity does not appear to affect the overall degree of matrix contraction measured at day-7.



1% NGM | **1% NGM + MMPI added at T4 Specific to gel contraction** | **1% NGM + TGF-β1** | **1% NGM + TGF-β1 + MMPI added at T0 Specific to TGF-β1 treatment**

Figure 6.8A Typical contraction of collagen matrices seeded with normal scar fibroblasts at day-7 of matrix culture. Cells were maintained in 1% NGM or 1% NGM + TGF-β1 with or without the addition of Ilomastat (MMPI) at 5μM. Ilomastat was added at day-4, on gel release or day-0 (24hrs after seeding the gels) in the presence of TGF-β1.

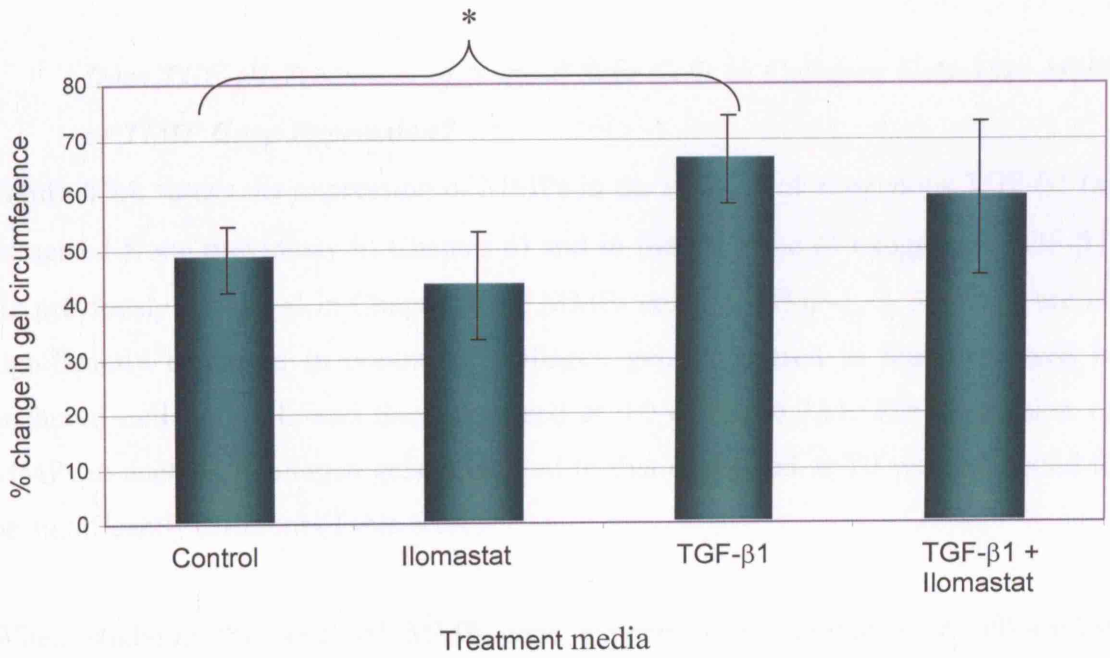


Figure 6.8B Graph to show the mean percentage change in gel circumference to that of the perimeter of the tissue culture well (original gel circumference). The degree of contraction was measured at day-7 of collagen matrix culture. Normal scar cells were maintained in 1% NGM with or without the addition of TGF-β1 at 2ng/ml and in the presence or absence of Ilomastat (5μM) added at day-4 (on gel release) or day-0 (in the presence of TGF-β1). Error bars represent SD. T-test analysis compared the percentage change in gel circumference after each different treatment media compared to 1% NGM alone. *P=<0.05.

6.2.2.2 Is the Secretion of MMPs and TIMPs Affected by Exogenous TGF-β1

From the results presented in section 6.2.2.1, it remains inconclusive as to whether MMPs are involved in exogenous TGF-β1-induced apoptosis in collagen gels. In order to further explore this possibility the expression pattern of MMPs and TIMPs after the addition of TGF-β1 to adherent collagen gels was compared with the pattern of expression seen after collagen gel contraction.

MMP and TIMP gene expression levels were assessed by RT-PCR from cells maintained in anchored and contractile collagen matrices, cultured in 1% NGM with or without the addition of exogenous TGF-β1. The level of gene expression was worked out as a ratio to that of the house-keeping gene GAPDH. MMP and TIMP gene expression were studied in both normal scar and keloid scar cell-seeded matrices in order to determine if any changes in gene expression patterns were common to both cell types, since both respond to TGF-β1-induced apoptosis in collagen gels.

Does TGF-β1 Treatment of Normal Scar Cells in Collagen Gels Alter MMP or TIMP Gene Expression?

Figure 6.9A shows the expression of MMPs in the absence of exogenous TGF-β1 (as presented alone previously in Chapter 4) and in the presence of exogenous TGF-β1. As previously discussed in Chapter 4 the MMPs studied, MMP-1, 2, 3 and 13 are all significantly increased in contractile collagen gels compared to that expressed in anchored collagen gels and that expressed at T0 (Table 6.2A). The expression of MMPs in anchored collagen gels compared to that expressed at T0 was not found to be significantly different (Table 6.2A).

When studying the level of MMP gene expression in normal scar cell-seeded anchored collagen matrices, it was found that in the presence of exogenous TGF-β1, although MMP-1 and 2 levels had increased they did not reach statistical significance when compared to that expressed at T0 or that expressed in anchored gels in the absence of TGF-β1 (Table 6.2A). When comparing the expression of MMP-1 and MMP-2 in contractile gels to that of anchored gels + TGF-β1, no significant difference was detected, whereas MMP-3 and -13 were significantly lower in TGF-β1-treated anchored gels.

The addition of exogenous TGF-β1 to normal scar cell-seeded contractile collagen gels caused a significant increase in MMP-1, MMP-2 and MMP-3 (p=0.013, 0.006 and 0.031, respectively) gene expression compared to that expressed at T0. In comparison to the gene levels expressed in contractile gels in the absence of TGF-β1, the addition of TGF-β1 to the contractile gels only significantly (p=0.020) affected MMP-2, where a ~1.5-fold increase in expression was detected (Figure 6.9A and Table 6.2). The gene expression levels of the other MMPs studied where not significantly affected by TGF-β1 (Figure 6.9A and Table 6.2).

MMP Gene Expression Levels

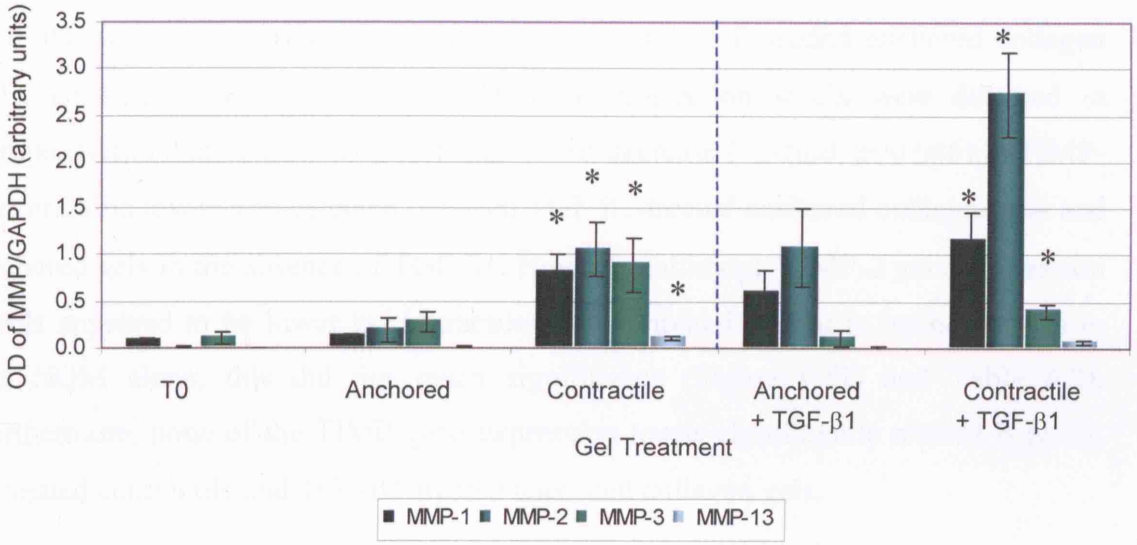


Figure 6.9A Densitometry analysis of RT-PCR results. Analysis of the RNA gene expression levels of MMP-1, -2, -3 and -13 from day-0 (T0-24hrs after seeding gels and prior to adding media) and day-7 anchored and contractile collagen matrices seeded with normal scar fibroblasts. Cells were cultured in 1% NGM with or without TGF-β1 at 2ng/ml. Results represent the expression of the gene of interest as a ratio to that of GAPDH. Results represent the experiment carried out with n=8 normal scar cell strains. Error bars represent SEM. T-test analysis compared the expression of the MMP after the different gel treatments to that expressed at T0. *P=<0.05.

The expression levels of TIMPs in normal scar cell-seeded matrices, maintained in 1% NGM alone (presented previously in Chapter 4) show that TIMP-1 and -2 is significantly (p=0.021 and 0.008, respectively) increased by day-7 in contractile collagen gels, compared to that expressed at T0 (Figure 6.9B and Table 6.2). TIMP-1 and -2 expression is increased in contractile gels by ~12-fold and 3.5-fold, respectively compared to that expressed at T0. TIMP-3 and -4 were not significantly

affected by contractile collagen gel culture. Normal scar cell-seeded, anchored collagen matrices also showed a significant (p=0.009) increase in TIMP-2 by day-7 compared to that at T0 (Figure 6.9B and Table 6.2). However, no significant difference in TIMP-1 or TIMP-2 expression was found between anchored and contractile gels. As well as TIMP-2 being significantly increased in anchored matrices, T-test analysis of TIMP-4 expression levels also detected a significant increase compared to that expressed at T0; however expression levels were barely detectable (Figure 6.9B and Table 6.2) and so the significance of this finding is unclear.

In contrast to results analysing the gene expression level of TIMPs in the absence of TGF-β1, with the addition of TGF-β1 to normal scar cell-seeded anchored collagen gels, no significant increase in TIMP gene expression levels were detected in comparison to that detected at T0. A significant decrease (~1-fold, p=0.046) in TIMP-2 expression levels was detected between TGF-β1-treated anchored collagen gels and anchored gels in the absence of TGF-β1. However, although TIMP-2 gene expression levels appeared to be lower in contractile gels compared to that in anchored gels in 1% NGM alone, this did not reach significance (Figure 6.9B and Table 6.2). Furthermore, none of the TIMP gene expression levels significantly altered between untreated contractile and TGF-β1-treated anchored collagen gels.

As with the anchored collagen matrices seeded with normal scar cells, TIMP-2 expression in contractile collagen matrices was also significantly (p=0.011) reduced in the presence of exogenous TGF-β1, compared to that seen in the absence of exogenous TGF-β1 (Figure 6.9B and Table 6.2). The other TIMPs studied were not significantly affected by the presence of exogenous TGF-β1.

In summary, the main differences between anchored and contractile collagen gels with regards to the expression level of MMP and TIMP genes appears to be a significant increase in all MMP expression levels and no significant change in TIMPs. In contrast, the main difference between untreated and TGF-β1-treated anchored gels was that although there appeared to be an increased expression of MMP-1 and -2 this did not reach significance. Comparison between TGF-β1-treated anchored matrices

and untreated contractile matrices also found no significant difference in the expression of MMP-1 and -2. Interestingly, TGF-β1-treatment of anchored gels significantly decreased the expression of the TIMP-2 gene, potentially indicating a mechanism by which MMP activity could also be increased.

Indeed, further quantitative analysis of MMP and TIMP mRNA expression needs to be carried out (for example, quantitative RT-PCR) for conclusive evidence. However, results from normal scar cells appear to imply that TIMP-2 and perhaps MMP-1 and MMP-2 may play roles in the apoptosis induced in collagen gels.

TIMP Gene Expression Levels

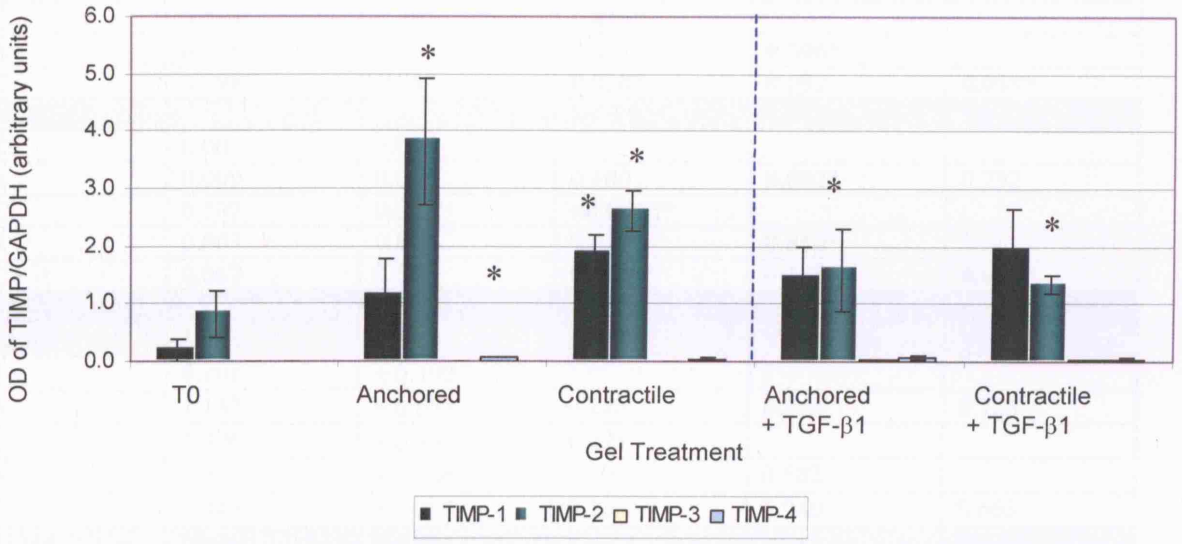


Figure 6.9B Densitometry analysis of RT-PCR results. Analysis of the RNA gene expression levels of TIMP-1, -2, -3 and -4 from day-0 (T0-24hrs after seeding gels and prior to adding media) and day-7 anchored and contractile collagen matrices seeded with normal scar fibroblasts. Cells were cultured in 1% NGM with or without TGF-β1 at 2ng/ml. Results represent the expression of the gene of interest as a ratio to that of GAPDH. Results represent the experiment carried out with n=8 normal scar cell strains. Error bars represent SEM. T-test analysis compared the expression of the TIMP after the different gel treatments to that expressed at T0. *P=<0.05.

Table 6.2 Results from T-test analysis of raw data assessing the expression of MMPs and TIMPs by RT-PCR in normal scar cell-seeded collagen gels. Cells were maintained in contractile (C) or anchored gels (A), with either 1% NGM or 1% NGM +TGF-β (+). T-test analysis was carried out on n=8 cell strains for both scar types.

Normal Scar			P-value		
MMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.076	0.039			
A	0.129	0.012	0.656	**0.017***	0.838
C	0.811	0.208	**0.015***		
A+	0.611	0.237	0.591	**0.018***	
C+	1.160	0.309	**0.022***	0.148	**0.031***
MMP-2			**Vs T0**	**Vs C**	**Vs A+**
T0	0.011	0.01			
A	0.392	0.203	1.0	**<0.001****	0.566
C	1.304	0.538	**<0.001****		
A+	1.021	0.439	0.586	**<0.001****	
C+	2.478	0.599	0.050	0.834	0.052
MMP-3			**Vs T0**	**Vs C**	**Vs A+**
T0	0.107	0.06			
A	0.281	0.105	0.356	**0.005***	0.356
C	0.894	0.285	**0.013***		
A+	0.108	0.08	0.402	**0.006***	
C+	0.398	0.082	**0.030***	0.692	**0.019***
MMP-13			**Vs T0**	**Vs C**	**Vs A+**
T0	0.00	0.00			
A	0.009	0.006	0.160	**0.003***	0.732
C	0.107	0.02	**<0.001****		
A+	0.003	0.025	0.553	**0.010***	
C+	0.067	0.021	**0.001****	0.128	**0.005***
Normal Scar			**P-value**		
TIMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.201	± 0.192			
A	1.147	± 0.633	0.227	0.365	0.723
C	1.876	± 0.33	0.021		
A+	1.475	± 0.506	0.107	0.582	
C+	1.946	± 0.707	0.106	0.940	0.663
TIMP-2	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.820	± 0.412			
A	3.824	± 1.097	**0.009***	0.223	**0.046***
C	2.260	± 0.354	**0.008***		
A+	1.089	± 0.813	0.571	0.112	
C+	1.592	± 0.38	0.102	**0.011***	0.978
TIMP-3	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.004	± 0.004			
A	0.000	± 0	0.374	0.374	0.064
C	0.003	± 0.003	0.904		
A+	0.034	± 0.011	0.091	0.058	
C+	0.031	± 0.003	**0.008***	0.219	0.380
TIMP-4	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.001	± 0.001			
A	0.070	± 0.014	**0.005***	0.08	0.521
C	0.049	± 0.025	0.120		
A+	0.074	±0.031	0.058	0.186	
C+	0.049	± 0.018	**0.039***	0.299	0.336

Does TGF-β1 Treatment of Keloid Scar Cells in Collagen Gels Cause Increased MMP Gene Expression or Decreased TIMP Gene Expression?

Figure 6.10 shows the expression of MMPs in the absence of exogenous TGF-β1 (presented previously in Chapter 4) and in the presence of exogenous TGF-β1. As previously discussed in Chapter 4 the MMPs studied, MMP-1, 2, 3 and 13 were all significantly increased in contractile collagen matrices compared to that expressed at T0 and in anchored collagen gels (Figure 6.10A and Table 6.3). The expression of MMPs in anchored collagen gels was not significantly different to that expressed at T0 (Table 6.3).

MMP Gene Expression Levels

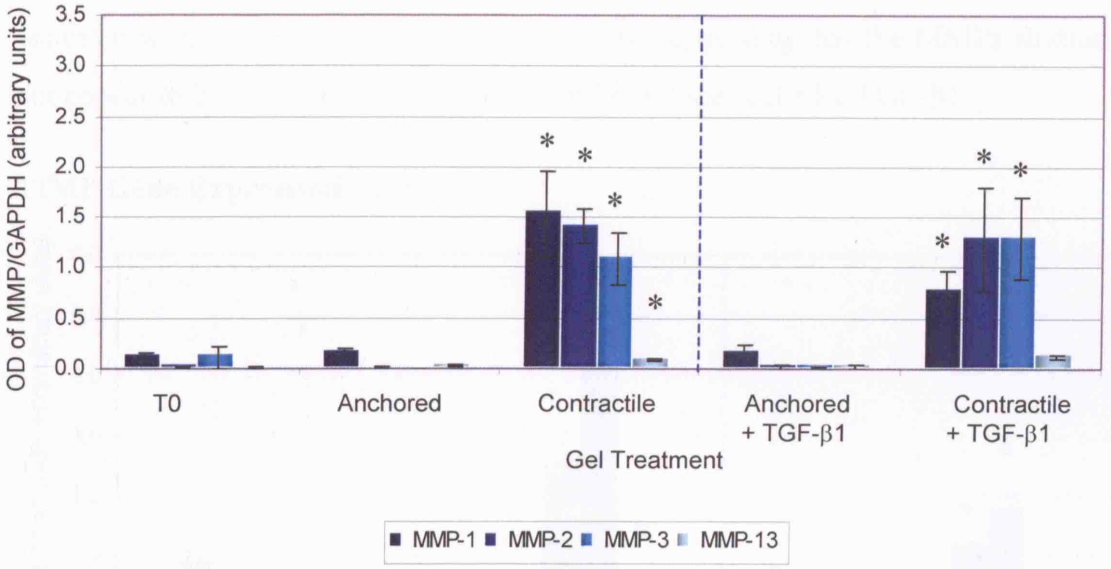


Figure 6.10A Densitometry analysis of RT-PCR results. Analysis of the RNA gene expression levels of MMP-1, -2, -3 and -13 from day-0 (T0-24hrs after seeding gels and prior to adding media) and day-7 anchored and contractile collagen matrices seeded with keloid scar fibroblasts. Cells were cultured in 1% NGM with or without TGF-β1 at 2ng/ml. Results represent the expression of the gene of interest as a ratio to that of GAPDH. Results represent the experiment carried out with n=8 keloid scar cell strains. Error bars represent SEM. T-test analysis compared the expression of the MMP after the different gel treatments to that expressed at T0. *P=<0.05.

Interestingly, the addition of TGF-β1 to keloid scar cell-seeded collagen matrices had no significant effect on the level of MMP gene expression compared to that detected in the absence of TGF-β1 (Figure 6.10A and Table 6.3). Regardless of whether TGF-β1 was present, MMP gene expression levels in contractile collagen gels remained elevated compared to that in anchored collagen gels.

The gene expression level of TIMPs expressed by keloid scar cells maintained in collagen matrices in 1% NGM alone has already been discussed in Chapter 4. Nevertheless, to reiterate for comparison, the expression levels of TIMP-1, -2 and -3 were significantly increased in contractile gels compared to that expressed both at T0 and in anchored gels (Figure 6.10A and Table 6.3). As with the expression level of MMPs the addition of TGF-β1 had no significant affect on the gene expression of TIMPs in anchored gels. However, the addition of TGF-β1 to contractile gels did significantly decrease the gene expression levels of both TIMP-1 and TIMP-2 (p=0.047 and 0.026, respectively).

Results of the gene expression levels of MMPs and TIMPs by keloid scar cells appears to show that there is no obvious pattern of MMP/TIMP expression in common with normal scar cell results, possibly suggesting that the MMPs studied do not appear to have a role in the apoptosis of keloid scar cells by TGF-β1.

TIMP Gene Expression Levels

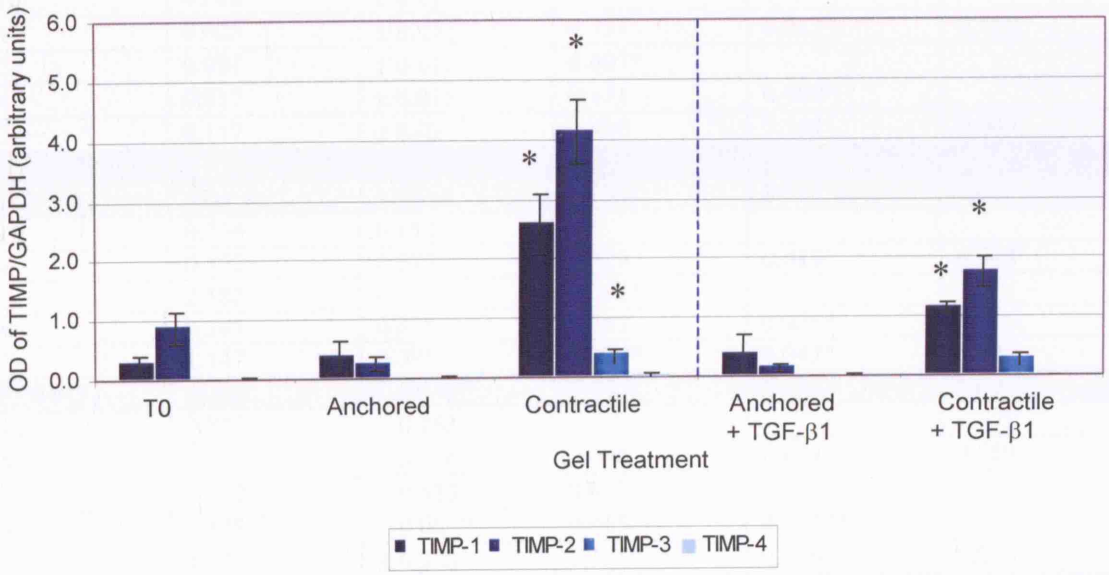


Figure 6.10B Densitometry analysis of RT-PCR results. Analysis of the RNA gene expression levels of TIMP-1, -2, -3 and -4 from day-0 (T0-24hrs after seeding gels and prior to adding media) and day-7 anchored and contractile collagen matrices seeded with keloid scar fibroblasts. Cells were cultured in 1% NGM with or without TGF-β1 at 2ng/ml. Results represent the expression of the gene of interest as a ratio to that of GAPDH. Results represent the experiment carried out with n=8 keloid scar cell strains. Error bars represent SEM. T-test analysis compared the expression of the TIMP after the different gel treatments to that expressed at T0. *P=<0.05.

Table 6.3 Results from T-test analysis of raw data assessing the expression of MMPs and TIMPs by RT-PCR in keloid scar cell seeded collagen gels. Cells were maintained in contractile (C) or anchored gels (A), with either 1% NGM or 1% NGM +TGF-β (+). T-test analysis was carried out on n=8 cell strains for both scar types. *P=<0.05 and **P=<0.001.

Keloid Scar			P-value		
MMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.118	± 0.05			
A	0.150	± 0.04	0.242	**0.017***	0.089
C	1.535	± 0.42	**0.013***		
A+	0.167	± 0.065	0.068	0.550	
C+	0.763	± 0.205	**0.013***	0.386	0.209
MMP-2	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.012	± 0.01			
A	0.009	± 0.005	0.205	**0.035***	0.086
C	1.408	± 0.165	**0.011***		
A+	0.021	± 0.02	0.061	0.698	
C+	1.285	± 0.52	**0.006***	**0.020***	**0.034***
MMP-3	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.112	± 0.11			
A	0.000	± 0	0.207	**0.046***	0.241
C	1.094	± 0.255	**0.038***		
A+	0.010	± 0.01	0.989	**0.038***	
C+	1.294	± 0.4	**0.031***	0.154	**0.045***
MMP-13	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.008	± 0.005			
A	0.025	± 0.011	0.334	**0.013***	0.560
C	0.081	± 0.011	**0.007***		
A+	0.017	± 0.015	0.151	**0.009***	
C+	0.117	± 0.02	0.430	0.341	0.065
Keloid Scar			**P-value**		
TIMP-1	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.256	0.152			
A	0.373	0.275	0.729	**0.019***	0.984
C	2.582	0.5	0.012*		
A+	0.383	0.318	0.765	0.025*	
C+	1.147	0.095	0.009*	0.047*	0.123
TIMP-2	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.873	± 0.263			
A	0.247	± 0.116	0.098	**0.004***	0.589
C	4.122	± 0.535	**0.027***		
A+	0.128	± 0.06	0.085	0.001**	
C+	1.756	± 0.258	0.123	**0.026***	**0.003***
TIMP-3	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.000	± 0			
A	0.010	± 0.005	0.123	**0.035***	0.676
C	0.356	± 0.109	**0.032***		
A+	0.006	± 0.005	0.374	**0.034***	
C+	0.271	± 0.09	0.061	0.608	0.065
TIMP-4	**Mean**	**± SEM**	**Vs T0**	**Vs C**	**Vs A+**
T0	0.016	± 0.018			
A	0.009	± 0.009	0.374	0.374	0.419
C	0.040	± 0.04	1.0		
A+	0.013	± 0.007	0.869	0.967	
C+	0.004	± 0.004	0.507	1.0	0.374

In order to aid the comparison of keloid scar cells with normal scar cells Figure 6.11 plots the gene expression levels of MMP-1, MMP-2 and TIMP-2 (those indicated as potentially important) from normal scar cells versus that expressed by keloid scar cells.

Comparison of MMP and TIMP Changes in Normal Scar vs Keloid Scar Cells

Figure 6.11 shows comparative results of MMP-1, -2 and TIMP-2 gene expression from normal scar cells versus keloid scar cells, after 7-days in collagen gel culture. The graph depicting results from MMP-1 gene expression shows that normal scar cells express significantly increased levels of this MMP in contractile gels versus T0, MMP-1 levels are also elevated in both anchored and contractile gels in the presence of TGF-β1 versus T0. Keloid scar cells in addition, show significantly increased MMP-1 levels in contractile gels versus T0 and contractile + TGF-β1 versus T0 (Figure 6.11). In the presence of TGF-β1 normal scar cells show a trend of increasing MMP-1 expression in both anchored and contractile gels + TGF-β1, compared to the equivalent gel type in the absence of TGF-β1. Keloid scar cells do not show this pattern expressed by normal scar cells, this apparent difference between the two cell types does not reach statistical significance however (Figure 6.11).

A similar result is seen with the expression of MMP-2 in normal scar cell-seeded collagen matrices, where MMP-2 expression levels were significantly elevated in contractile gels. With the addition of TGF-β1, MMP-2 gene expression levels were at least equivalent to that seen in contractile gels, if not further increased. In contrast, the keloid scar fibroblasts showed only an increase in MMP-2 gene expression levels in contractile gels. As with MMP-1, MMP-2 expression was not affected by TGF-β1 in keloid scar cell-seeded matrices. Normal scar cells express significantly higher levels of MMP-2 in anchored matrices in both the absence ($p=0.029$) and the presence ($p= 0.029$) of TGF-β1 compared to that expressed by keloid scar cells (Figure 6.11). In addition normal scar cells also show a trend of increased MMP-2 expression in contractile gels + TGF-β1, compared with keloid scar cells (Figure 6.11).

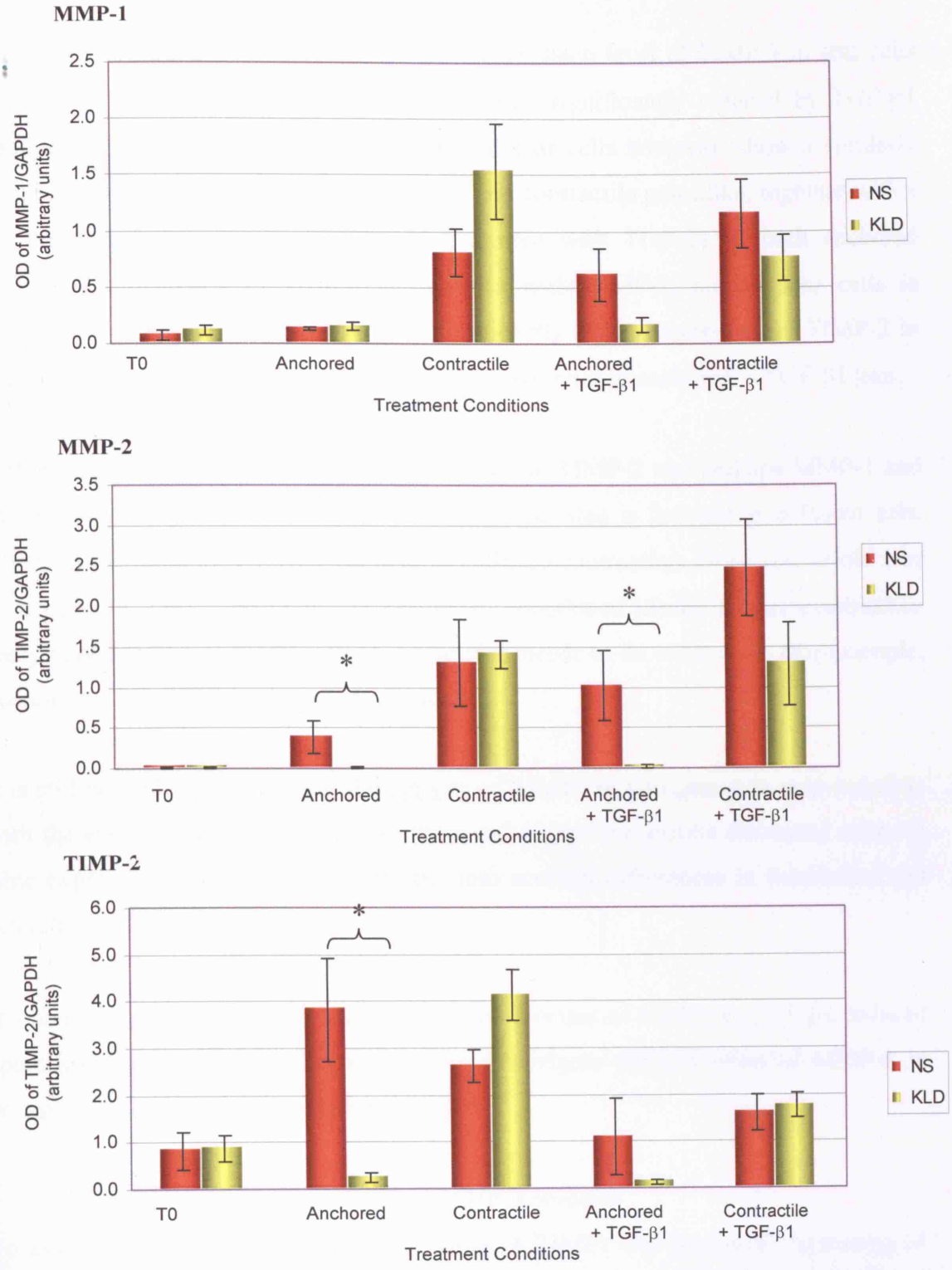


Figure 6.11 Comparative gene expression levels of MMP-1, MMP-2, and TIMP-2 between normal scar and keloid scar fibroblasts, after T0 (24 hrs after seeding the gels and prior to adding media) and 7-days of collagen gel culture. Cells were maintained within anchored or contractile matrices in 1% NGM with or without the addition of TGF-β1 (2ng/ml). Error bars represent SEM. T-test analysis compared the expression of the gene of interest by normal scar vs keloid scar cells for each different gel treatment *P=<0.05.

The graph in Figure 6.11 depicting the gene expression level of TIMP-2 in scar cells shows that for keloid scar cells TIMP-2 is only significantly reduced by TGF-β1 treatment in contractile collagen gels. Normal scar cells however, show a similarly high expression of TIMP-2 in anchored gels and contractile gels alike, together with a significant reduction in expression on treatment with TGF-β1 in both anchored ($p=0.046$) and contractile ($p=0.011$) gels. As with MMP-2, normal scar cells in comparison to keloid scar cells show a significantly higher expression of TIMP-2 in anchored ($p=0.007$) collagen gels and an increased trend in anchored + TGF-β1 gels.

Results with normal scar cells appear to show that TIMP-2 and perhaps MMP-1 and MMP-2 may play important roles in the apoptosis that is induced in collagen gels, whether it is through TGF-β1-treatment or collagen contraction. However, keloid scar cells show no such correlation. As previously mentioned ideally further quantitative analysis of MMP and TIMP mRNA expression needs to be carried out (for example, quantitative RT-PCR) for conclusive evidence.

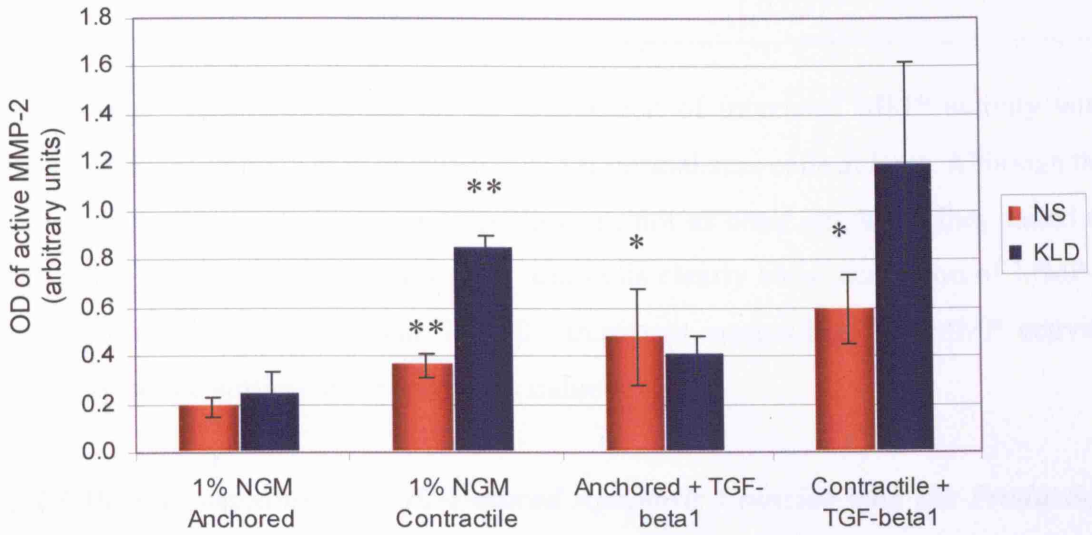
It is still possible however, that the activity of MMPs in collagen gels may correlate with the apoptosis that occurs within these gels; since the results discussed relate to gene expression alone and does not take into account differences in translation and activation of these proteins.

In order to further investigate the possible involvement of MMPs in TGF-β1-induced apoptosis, zymography was carried out to investigate the activation of MMP-2 in normal scar cells versus keloid scar cells.

TGF-β1-induced Apoptosis and MMP Activation

To assess whether the decreased expression of TIMPs and increased expression of MMPs correlates with increased MMP activation zymography was carried out to assess the active levels of MMP-2. The expression of active MMP-2 levels in anchored and contractile collagen matrices under the effects of minimal growth medium alone have already been presented in Chapter 4. Figure 6.12 displays these results again for comparison, and to reiterate shows a significant increase in active MMP-2 levels in contractile collagen gels compared to anchored collagen gels for both normal scar and, surprisingly, keloid scar cell-seeded matrices ($P=<0.001$ and

<0.001, respectively). Figure 6.12 also shows the effect of exogenous TGF-β1 on the levels of active MMP-2 in normal scar cell-seeded matrices, which significantly increased to an equivalent level in both anchored and contractile gels (p=0.03 and 0.016, respectively and Table 6.4). Keloid scar cells showed a similar trend of increasing MMP-2 activation, although this did not reach statistical significance. This is possibly due to the increased variability of active MMP-2 levels displayed by keloid scar cell strains.



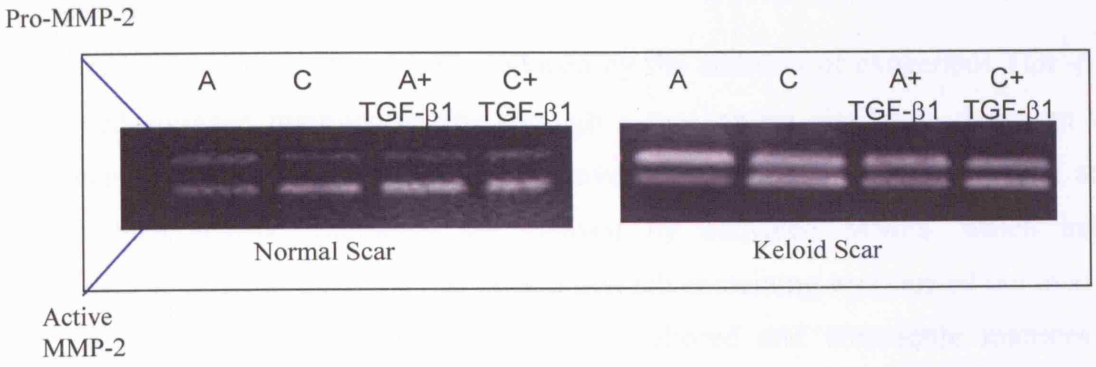


Figure 6.12 Densitometric analysis of Zymography results, representing the expression of active MMP-2. Zymography was carried out on conditioned medium from day-7 anchored and contractile collagen matrices seeded with either normal scar or keloid scar-derived fibroblasts. Cells were cultured in 1% NGM with or without the addition of TGF-β1 at 2ng/ml. Results are representative of n=4 cell strains for each scar type, carried out in duplicate. Error bars are SD. T-test analysis compared the level of active MMP-2 expressed by normal scar vs keloid scar after each gel treatment. **P=<0.001, *P=<0.05.

Table 6.4 Results from T-test analysis of raw data assessing the expression of active MMP-2 by zymography with normal scar and keloid scar cell conditioned medium from day-7 anchored (A) or contractile (C) collagen gels. Cells were maintained in 1% NGM or 1% NGM +TGF-β1 (+). T-test analysis was carried out on n=4 cell strains for both scar types. **P=<0.001, *P=<0.05.

Gel type and Media type	Normal Scar Cells	Keloid Scar Cells
A vs C	<0.001**	<0.001**
A vs A+	0.003*	0.129
C vs C+	0.016*	0.186
A+ vs C+	0.262	0.027*

These results provide evidence for an association of increased MMP activity with TGF-β1-induced apoptosis in collagen gels for normal scar cells at least. Although the results are similar for keloid scar cells they are not as clear cut, since they failed to reach significance. Nevertheless, keloid scar cells clearly show activation of MMP-2 during collagen contraction and TGF-β1 treatment suggesting that MMP activity alone may not be sufficient for apoptosis induction.

6.2.2.3 Does Exogenous TGF-β1-Induced Apoptosis Coincide with the Production of Small Polypeptide Fragments, as seen with Collagen Contraction-Induced Apoptosis?

The apoptosis of normal scar cells induced by the addition of exogenous TGF-β1 to anchored collagen matrices maybe through a mechanism similar to that seen with collagen contraction-induced apoptosis, involving MMPs. It is possible that small extracellular matrix fragments are cleaved by activated MMPs, which induce apoptosis. In order to examine this possibility, silver staining was carried out to assess the protein fragments produced in day-7 anchored and contractile matrices for comparison with anchored matrices in the presence of TGF-β1. Contractile gels + TGF-β1 were not studied, as the interest of this experiment was in the effect of TGF-β1 on anchored collagen gels versus contractile collagen gels in the absence of TGF-β1.

Cells were seeded into collagen gels and cultured for 7-days, maintained in 1% NGM with or without the addition of TGF-β1 at 2ng/ml. Matrices were either retained

anchored or released at day-4 and allowed to contract. At day-7 collagen matrices conditioned by normal scar or keloid scar cells were homogenised, and samples were prepared for SDS-PAGE analysis of protein degradation.

Silver Staining

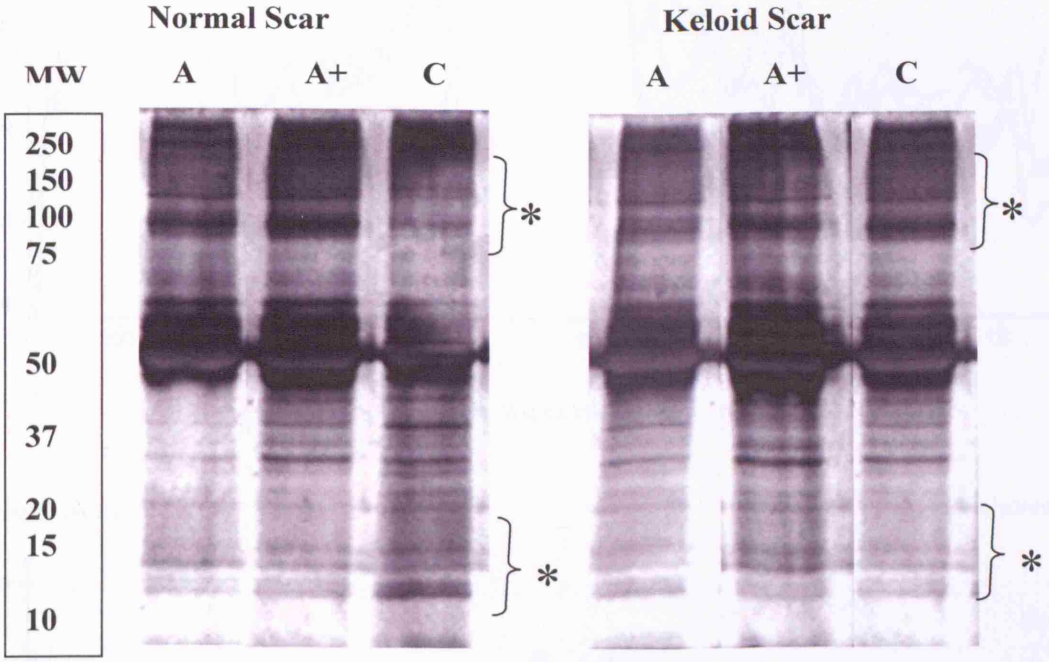
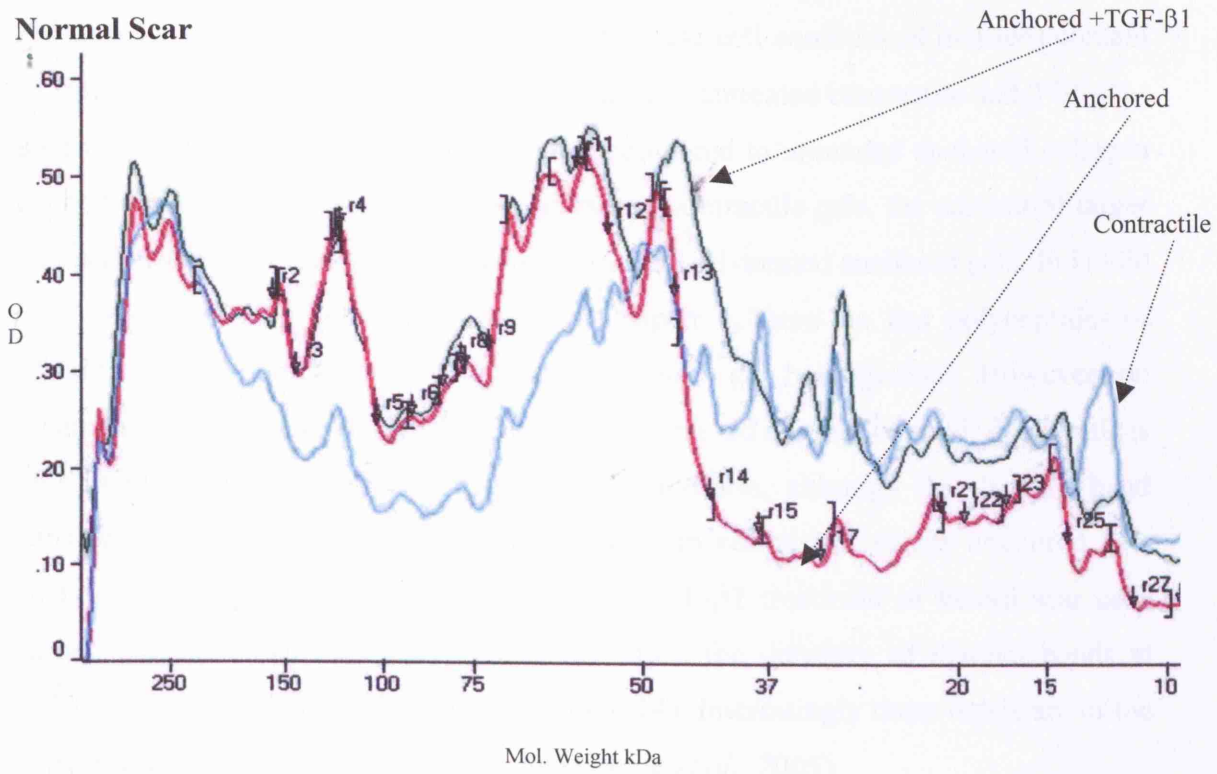


Figure 6.13 Typical silver staining of anchored (A) and contractile (C) day-7 collagen gel lysates after SDS-PAGE. Normal scar and keloid scar fibroblasts were cultured in collagen matrices maintained in either 1% NGM or 1% NGM + TGF-β1 (+) for 7-days. At which point the cells along with the collagen matrices were homogenised and lysed in SDS lysis buffer, then run on a 10% SDS gel. This experiment was repeated three times with n=4 normal scar and keloid scar cell strains. Asterix highlights areas regions of decreased or increased polypeptide species.



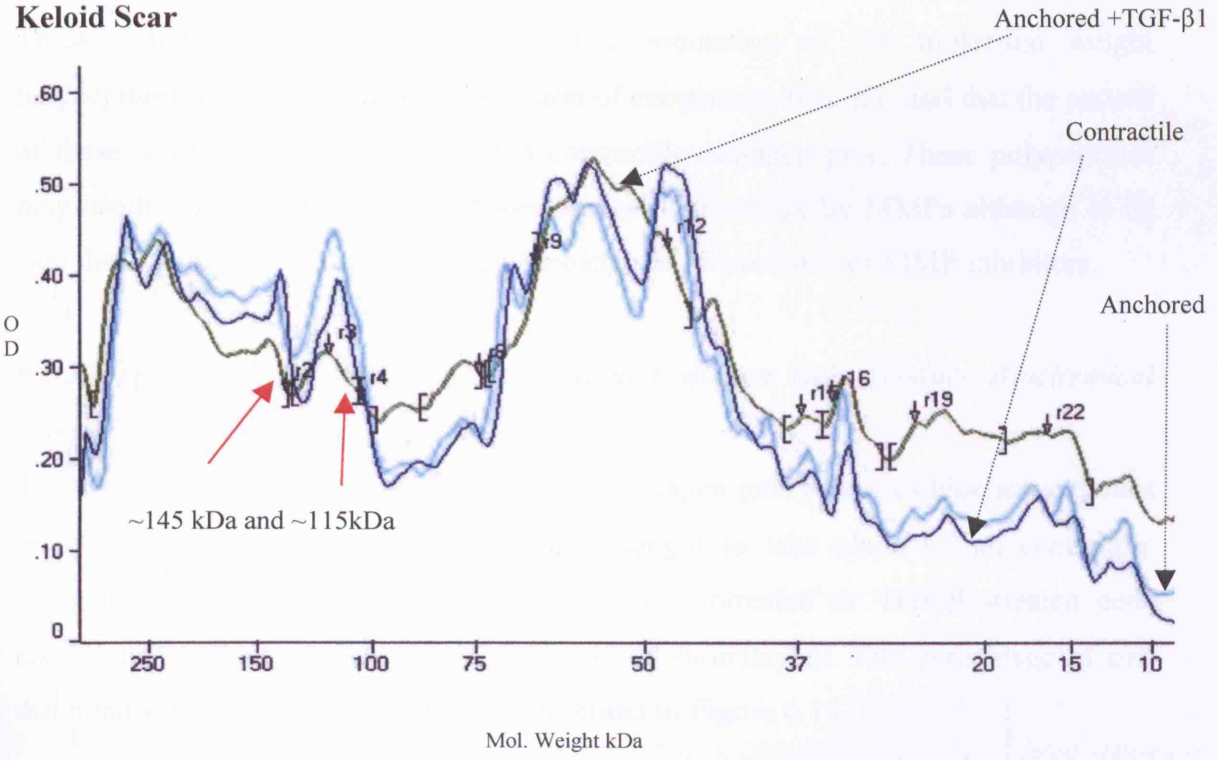


Figure 6.14 Densitometric analysis of silver stained collagen gel lysates after SDS-PAGE, analysing protein band size (kDa). The x-axis represents the optical density of each protein band. Protein lysates were from day-7 anchored and contractile collagen matrices conditioned by normal scar or keloid scar fibroblasts, maintained in 1% NGM with or without TGF-β1 at 2ng/ml. Brackets and arrows represent numbered peaks.

Figure 6.13 and 6.14 demonstrates that normal scar cell conditioned matrices contain an increased amount of polypeptides of <50kDa in untreated contractile and TGF-β1–treated anchored collagen gel homogenates compared to untreated anchored collagen gel homogenates. Interestingly, unlike untreated contractile gels, the amount of larger polypeptides of >50kDa does not decrease in TGF-β1-treated anchored gels. In keloid scar cell-seeded matrices, as presented in Chapter 4, there are few polypeptides of <50kDa in either anchored or contractile collagen gel homogenates. However, on treatment with exogenous TGF-β1 there is an increased amount of small polypeptides of <50kDa seen (Figure 6.13 and 6.14). In addition, although the drop in band densities between 50 and 150kDa that is seen in contractile versus anchored gels containing normal scar cells does not occur, TGF-β1 treatment of keloid scar cell-seeded anchored matrices does appear to reduce the densities of distinct bands at ~115kDa and ~145kDa (red arrows - Figure 6.14). Interestingly these bands are of the correct size for the α-subunits of collagen (Linge *et al.*, 2005).

These results demonstrate that there is a population of low molecular weight polypeptides that appears with the addition of exogenous TGF-β1, and that the pattern of these is similar to that seen within contractile collagen gels. These polypeptides may result from the breakdown of the extracellular matrix by MMPs although to be sure the experiment would need to be repeated in the presence of MMP inhibitors.

6.2.2.4 Does TGF-β1 Treatment of Cells in Collagen Gels Produce Biochemical Cues of Apoptosis?

To determine whether TGF-β1-treatment of collagen gels produces biochemical cues of apoptosis in a similar fashion to that thought to take place within contractile collagen gels, the effect of homogenates from untreated or TGF-β1-treated cell-conditioned collagen gels were studied on cell monolayers. The percentage of cell death induced by each homogenate is presented in Figure 6.15.

Figure 6.15 shows the effect of both normal scar and keloid scar cell-conditioned collagen gel homogenates on both normal scar and keloid scar cell monolayer cultures (part of which was previously presented in Chapter 4). Normal scar cell monolayer cultures not only undergo cell death in response to normal scar cell-conditioned

contractile collagen homogenates (p= <0.001), but also to TGF-β1-treated normal scar and keloid scar cell-conditioned anchored collagen gel homogenates (p= 0.004 and <0.001, respectively). Normal scar cell monolayer cultures do not however, respond to acellular collagen gel homogenates or normal scar cell-conditioned anchored collagen gel homogenates (both –ve controls) as well as keloid scar cell-conditioned contractile collagen gel homogenates. Surprisingly, not only did keloid scar cell monolayers respond to normal scar cell-conditioned contractile collagen gel homogenates (p=0.006, as presented in Chapter 4), but they also responded to TGF-β1-treated normal scar and keloid scar cell-conditioned anchored collagen gel homogenates (p=0.010 and 0.008, respectively) (Figure 6.15). Keloid scar cell monolayers, like normal scar cell monolayers underwent between 40-60% cell death with these homogenates. Importantly, no cell monolayer cultures responded to contractile collagen matrices conditioned by keloid scar cells.

Results presented in Figure 6.15 provide evidence that only normal scar cell-conditioned contractile collagen gels and TGF-β1-treated anchored collagen gels are capable of significantly inducing cell death, this correlates with the accumulation of small polypeptides seen in these gels. Cell monolayers, irrespective of scar origin responded in an equivalent degree to the cell conditioned gel homogenates (Figure 6.15); this is unlike the induction of apoptosis in response to collagen breakdown products or synthetic RGD-peptides (Chapter 4), where keloid scar cells did not undergo apoptosis.

The addition of exogenous TGF-β1 appears to eradicate the fault seen with keloid scar cells, which is further demonstrated in Figures 6.13 and 6.14, where there is an increase in the production of small polypeptide fragments with the addition of TGF-β1. These polypeptides as discussed previously are implicated in collagen contraction-induced apoptosis and possibly TGF-β1-induced apoptosis. It is possible that keloid scar cells have a fault in their autocrine expression or bioavailability of TGF-β1. The exogenous addition of this growth factor thus allows keloid scar fibroblasts to produce the necessary cues required for successful apoptosis induction. This may be through cell differentiation and activation as discussed in Chapter 5 leading to the production of apoptosis cues and/or the ability to respond to these apoptosis cues.

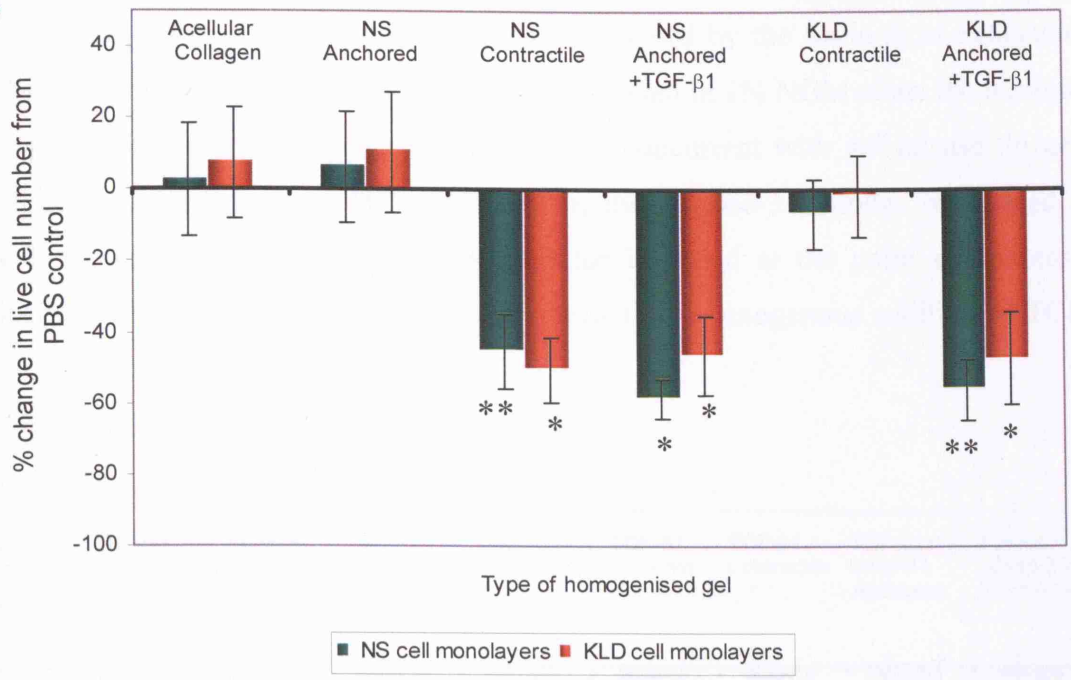


Figure 6.15 Effect of homogenised cell-conditioned collagen matrices on normal scar and keloid scar cell monolayer cultures. Collagen matrices were seeded with normal scar or keloid scar fibroblasts, cultured in 1% NGM with or without the addition of TGF-β1 at 2ng/ml. At day-4 the matrices were either retained anchored or released and allowed to contract for 3-days. At day-7 of matrix culture collagen matrices were homogenised before adding to monolayer cultures of either normal scar or keloid scar cells cultured on collagen coated (10μg/ml) 6-well plates. After 24hrs cell counts were performed with cell monolayers. This experiment was carried out in triplicate with n=4 normal scar and keloid scar cell strains. Error bars are SD. T-test analysis compared the effect of each homogenised gel solution vs the homogenised acellular collagen solution on the survival of cell monolayers. *P=<0.05, **P=<0.001.

6.2.3 Is Caspase-3 Involved in TGF-β1 Induced Apoptosis?

Results presented in this chapter demonstrate that the apoptosis cues produced by normal scar cells only, on the addition of exogenous TGF-β1 appear to mimic in many ways that which occurs during collagen contraction-induced apoptosis. Previously Chapter 3 demonstrated that collagen contraction-induced apoptosis involved the participation of caspase-3. Furthermore, the apoptosis induced by RGD-peptides (possible mechanism of apoptosis that takes place during collagen contraction) has been found to be through specific caspase-3 activation (Buckley *et al.*, 1999).

A specific inhibitor of caspase-3 was therefore used to investigate whether caspase-3 activation is required for the apoptosis that is induced by the addition of exogenous TGF-β1 to collagen gels. Where gels were maintained in 1% NGM alone the inhibitor was added at day-4 of collagen matrix culture, concurrent with gel release, in gels cultured in the presence of TGF-β1 however, the caspase-3 inhibitor was added at day-0. In this manner, the caspase-3 inhibitor is added at the point of apoptosis induction, whether it is through collagen contraction or exogenous addition of TGF-β1.

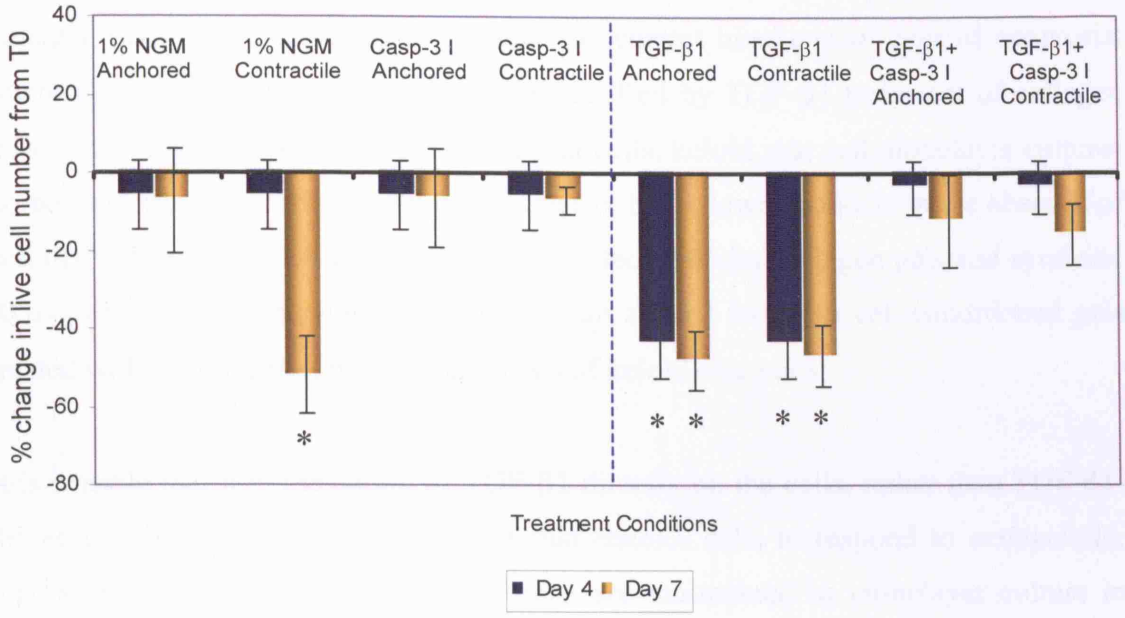


Figure 6.16 Collagen contraction-induced apoptosis with or without the addition of a specific caspase-3 inhibitor (Casp-3I) at 10μM. The inhibitor was added to the 1% NGM at day-4 in the absence of TGF-β1 or at day-0 (24hrs after seeding the gels) in the presence of TGF-β1. Normal scar-derived fibroblasts were seeded into collagen matrices and viable cell number assessed day-0, -4 and -7. These results represent the percentage change in live cell number at day-4 and -7 from that at day-0. The results represent the mean of the experiment carried out in triplicate with n=4 normal scar cell lines. Error bars are SD. T-test analysis compared the percentage change in live cell number after each different treatment medium at day-7 vs that at day-7 after treatment with 1% NGM alone.*P = <0.05.

Figure 6.16 shows that the addition of the caspase-3 inhibitor to normal scar cell-seeded matrices maintained in 1% NGM alone, inhibited the significant (p= 0.006) induction of apoptosis seen in contractile collagen gels. In addition, the significant (p= 0.006) induction of apoptosis induced by the addition of exogenous TGF-β1 to normal

scar cell-seeded matrices, was also abrogated, at both day-4 and day-7. The caspase-3 inhibitor appeared to have no significant effect on normal scar fibroblasts which were maintained in anchored collagen matrices with 1% NGM alone (Figure 6.16).

6.2.4 Does Active TGF-β1 Affect the Ability of Keloid Scar Cells to Respond to Products of Collagen Remodelling?

Both normal scar and keloid scar cell monolayers are able undergo apoptosis in response to normal scar cell-conditioned contractile collagen gels homogenates, they are however unable to respond to keloid scar cell-conditioned contractile collagen gel homogenates, this suggests a failure of keloid scar cells to condition a contractile collagen gel in such a way as to produce the correct biochemical cues of apoptosis. Interestingly, this apparent defect can be rectified by TGF-β1-treatment of collagen gels. In contrast however, unlike normal scar cells, keloid scar cell monolayer cultures do not undergo apoptosis in response to matrix breakdown products in the absence of cell derived factors, such as collagenase digested acellular collagen gels and synthetic RGD-peptides. This phenomenon suggests an altered factor in cell-conditioned gels treated with TGF-β1 that permits apoptosis of keloid scar cells.

It is feasible that it is the action of TGF-β1 directly on the cells, rather than TGF-β1-driven changes to the 3D-environment that enables cells to respond to extracellular matrix fragments. When normal scar cells are maintained in monolayer culture in serum free culture conditions, these stressful conditions may signal autocrine TGF-β1. Keloid scar cells however, may have an aberrant expression/activation of autocrine or altered bioavailability of TGF-β1 (through sequestration by diverse TGF-β-binding factors), therefore rendering the cells unable to respond to apoptosis cues.

For this reason the effect of extracellular breakdown products was studied on keloid scar fibroblasts in the presence and absence of TGF-β1. Acellular collagen gels were digested with a collagenase D solution, this solution was size fractionated by filtration and protein fragments of <10kDa used. The effect of this solution containing small extracellular matrix fragments in the presence of protease inhibitors with or without TGF-β1 at 2ng/ml was tested on keloid scar fibroblast monolayer cultures, along with the collagenase enzyme solution alone as a control. Results displayed in Figure 6.17

shows the effect of this polypeptide solution. After 48hrs, in the absence of TGF-β1 cells appear healthy (in agreement with results previously presented in Chapter 4) however; in the presence of TGF-β1 the digested collagen gel solution did induce significant cell death in keloid scar cell monolayer cultures. This was determined by the rounded-up unhealthy appearance of the cells (Figure 6.17). The solution of collagenase alone had no effect on keloid scar cells in the presence or absence of TGF-β1. The cell death detected therefore appears to be specific to the digested collagen gel solution. TdT end-labelling was not carried out on these cells due to difficulties in removing the active enzyme solution and adding the TdT-end labelling solution. The dying cells had detached from the base of the dishes and as such would have been discarded with the removal of the enzyme solution.

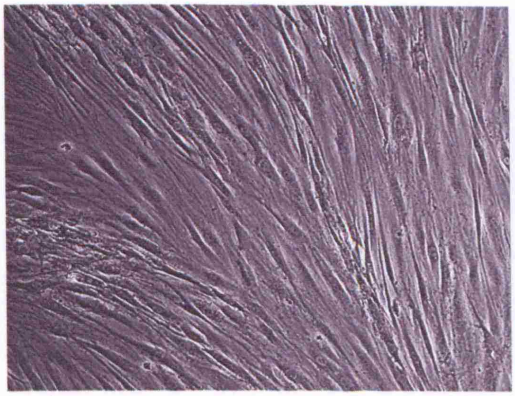
As previously discussed in Chapter 4, RGD-motif containing peptides are proposed to be the most likely peptides produced during collagen contraction/remodelling that can induce fibroblast apoptosis. To assess whether these peptides are also effective in inducing apoptosis of keloid scar fibroblast monolayer cultures in the presence of exogenous TGF-β1, a similar assay was set up, but with synthetic RGD-peptides and RAD-peptides (negative control) rather than enzymatically produced breakdown products of collagen gels.

Figure 6.18 shows that in agreement with that previously presented in Chapter 4, keloid scar fibroblast monolayers did not undergo apoptosis in responce to RGD-peptides. However, in the presence of TGF-β1, synthetic RGD-peptides and not RAD-peptides (control) are effective in inducing apoptosis, as demonstrated by the green apoptotic nuclei; detected using TDT end-labelling of cells (Figure 6.18).

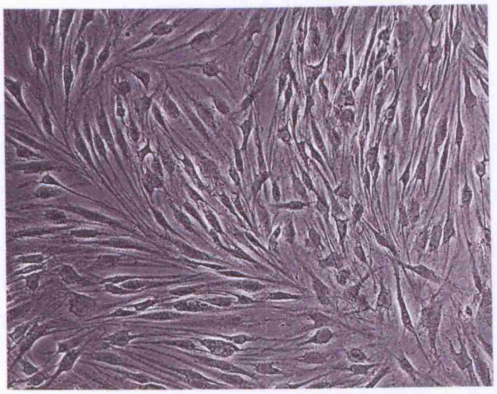
These results confirm that with the addition of TGF-β1, keloid scar fibroblasts are able to respond to products of extracellular matrix remodelling that act as biochemical cues of apoptosis.

Keloid scar fibroblast monolayers

Collagenase enzyme solution + Inhibitors

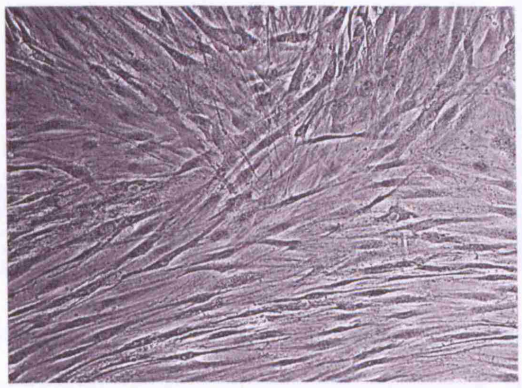


Collagenased collagen gel solution + Inhibitors



Keloid scar fibroblast monolayers + TGF-β1

Collagenase enzyme solution + Inhibitors



Collagenased collagen gel solution + Inhibitors

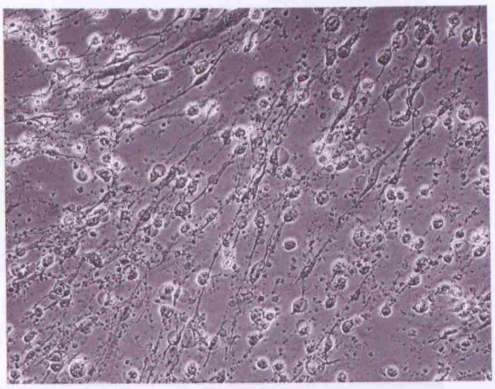
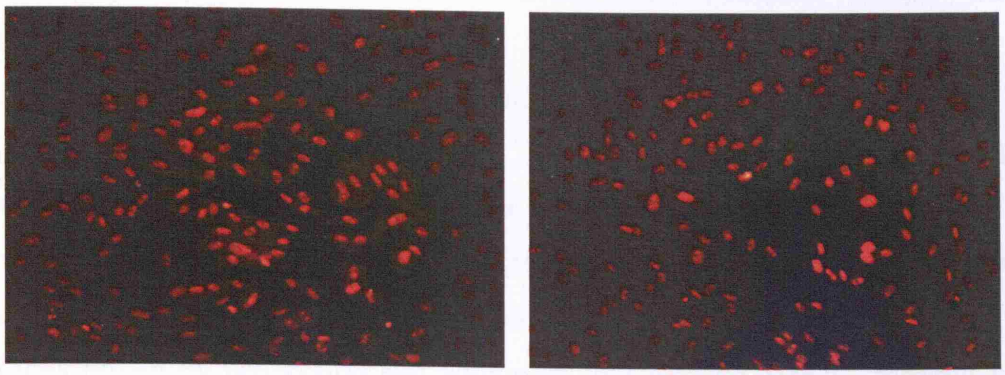
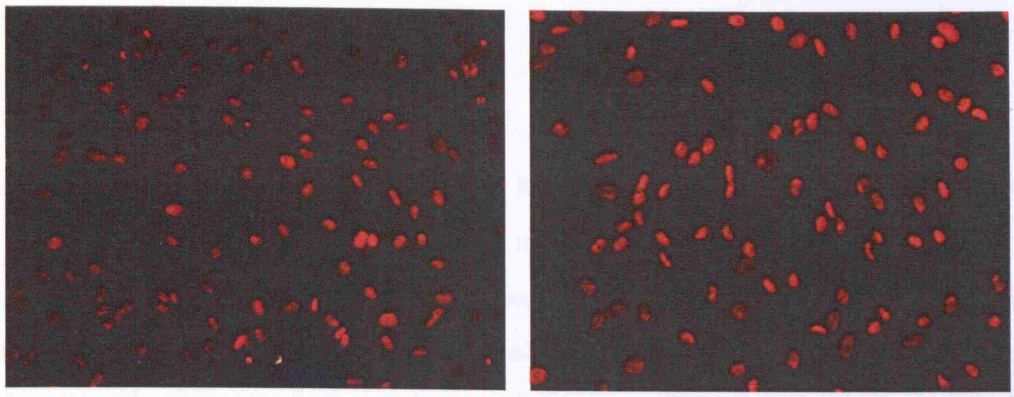


Figure 6.17 Typical micrographs of keloid scar cell morphology after treatment with a collagenase digested solution of acellular collagen gels. Keloid scar cells were cultured on collagen coated (10μg/ml) monolayers in SFM for 48hrs. Media was then replaced with a solution of digested acellular collagen gels with or without the addition of TGF-β1. That is cell-free collagen matrices digested with a collagenase-D solution which is filtered (<10kDa MWCO) and then Aprotinin (100IU/ml) added. The collagenase-D solution with Aprotinin was used alongside as a control. Cell death was assessed after 48hrs of treatment. This result is representative of n=4 keloid scar strains. (x200 Mag).

Keloid scar fibroblast monolayers treated with 1mM RGD peptides



Keloid scar fibroblast monolayers + TGF-β1 treated with 1mM RAD peptides



Keloid scar fibroblast monolayers + TGF-β1 treated with 1mM RGD peptides

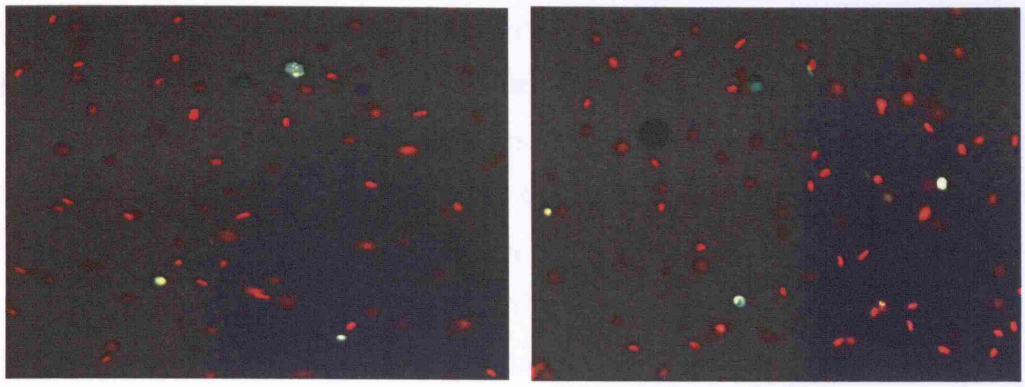


Figure 6.18 Typical terminal deoxynucleotidyl transferase end-labelling of keloid scar fibroblasts treated with 1mM of synthetic RGD or RAD (negative control) peptides in SFM for 48 hours. Keloid scar fibroblasts were treated with these peptide solutions in the presence or absence of TGF-β1 at 2ng/ml. Cells were initially seeded onto collagen coated (10μg/ml) glass-cover slips in 6-well tissue culture plates in SFM, after 48hrs the culture media was refreshed with a solution of RAD-peptides or RGD-peptides. Cell death was assessed after 48hrs of treatment. This result is representative of n=5 keloid scar cell strains. (x100 Mag).

6.2.5 Is the TGF-β1-driven Change in Capability of Keloid Scar Cells to Respond to the Apoptosis Signals of Matrix Breakdown Products Simply due to Myofibroblast Differentiation?

It is not conclusive yet from the results presented in Chapter 5 as to whether cells have to be of the myofibroblast phenotype to respond to collagen contraction/remodelling cues of apoptosis. It is possible that the results given in Figure 6.15 could be explained by changes in myofibroblast differentiation: where normal scar cells might be able to differentiate into myofibroblasts under these conditions driven by processing and activation of autocrine TGFβ1, whereas keloid cells may not be able to differentiate into myofibroblasts (as seen during anchored collagen gel culture – Figure 5.8) due to faulty production or activation of autocrine TGFβ1. This would be salvaged by the addition of exogenous active TGF-β1.

In order to investigate this hypothesis, the RGD peptide experiments in section 6.2.4 were repeated in an identical fashion but in the absence of the peptides, where the proportion of myofibroblast differentiation of the different scar cell types, under different conditions was assessed. Specifically, fibroblasts from both normal scar and keloid scar cell strains were seeded onto collagen type-I coated plastic in SFM and allowed to settle over a 48hr period (sufficient time to allow cells to adhere to the collagen substrate and spread out). At this point, when RGD peptides in the presence or absence of TGF-β1, in PBS were normally added, PBS alone or PBS + TGF-β1 were added to the cell monolayer cultures. The cells were incubated for a further 48hrs (same time-period as used in Figure 6.17 and 6.18) and then fixed and stained for α-SMA. The results are shown in Figure 6.19, which show that neither keloid scar or normal scar fibroblasts exhibited significant myofibroblast differentiation in the absence of TGF-β1. Treatment of cells with TGF-β1 for 48hrs did result in the appearance of myofibroblasts, but these were relatively few (less than 5%) with no obvious difference between the two scar cell types.

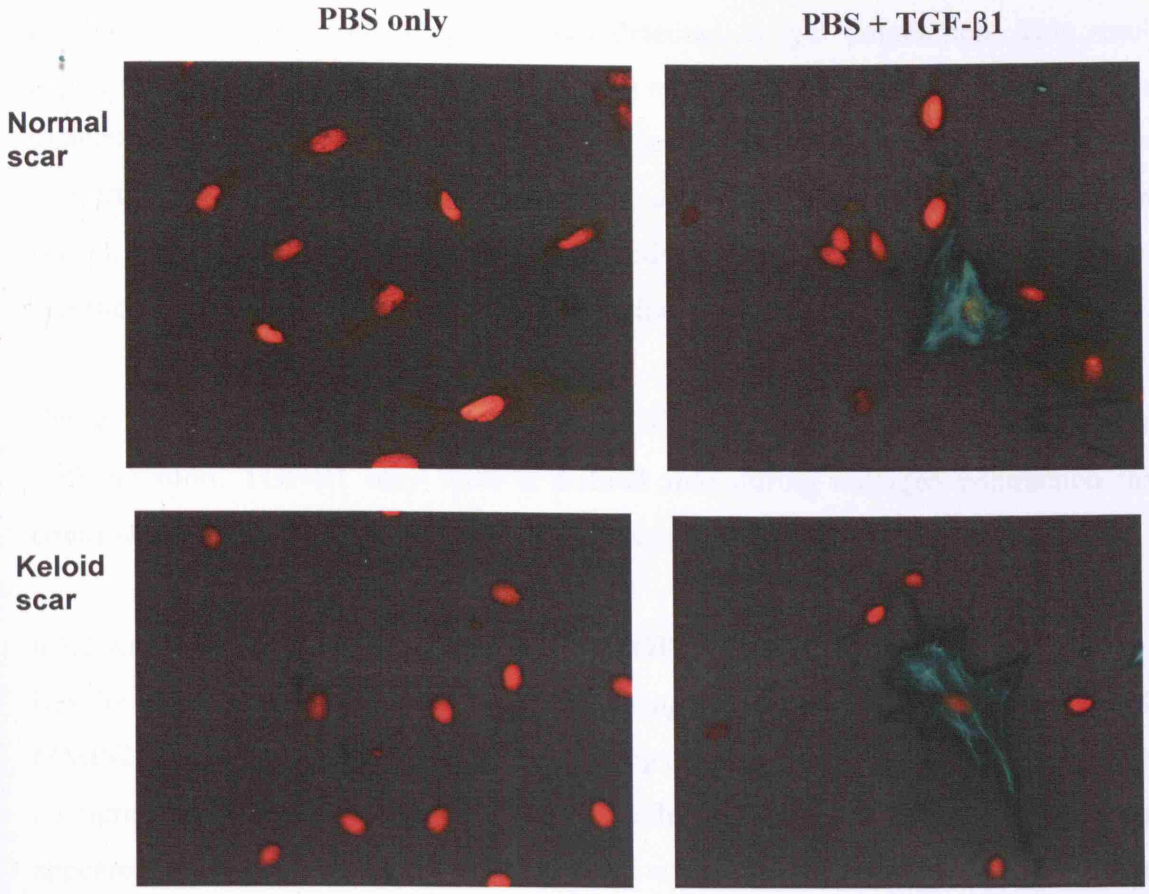


Figure 6.19 Typical α-SMA staining of normal scar and keloid scar cell monolayer cultures treated with and without TGF-β1. Cells were seeded onto collagen-coated plastic and maintained in SFM for 48hrs prior to replacing medium with PBS or PBS + TGF-β1 for a further 48hrs. Cells were then fixed for α-SMA detection. This result is representative of the experiment carried out in duplicate with n=3 cell strains for each scar type. (x200 Mag).

6.3 Discussion

Chapter 5 clearly demonstrated that TGF-β1 could induce fibroblast apoptosis; and that this was context dependant being specific to a 3-D collagen matrix, similar to that of collagen contraction-induced apoptosis. It was therefore hypothesised that TGF-β1 could in some way be involved with collagen contraction-induced apoptosis.

6.3.1 Is TGF-β1's Effects Simply Through Myofibroblast Differentiation?

Results presented in this chapter demonstrate that with the addition of TGF-β1 blocking agents at T0 (beginning of collagen matrix culture), myofibroblast differentiation is not able to take place in normal scar cell-seeded anchored collagen

matrices. In addition, no apoptosis was detected on gel contraction. This result appears to corroborate earlier suggestions that only myofibroblasts are able to respond to apoptosis cues. However, further investigation argued against this possibility; since with the addition of TGF-β1 blocking agents at day-4 of the collagen matrix culture period, normal scar cells differentiated into activated myofibroblasts (as judged by the presence of α-SMA) yet apoptosis seen on collagen contraction was again inhibited.

These results demonstrate that in addition to TGF-β1's role of inducing myofibroblast differentiation, TGF-β1 may have a distinct role during collagen contraction that contributes towards the induction of fibroblast apoptosis.

6.3.2 Are MMPs and TIMPs Involved in TGF-β1-Induced Apoptosis?

Results from blocking a range of MMPs using Ilomastat and blocking specifically MMP-2 produced contrasting results. Blocking a broad set of MMPs appeared to have no significant affect on inhibiting TGF-β1-induced apoptosis. In fact, the cell death appeared to be exaggerated; although this did not appear to be simply through toxicity (as demonstrated by a toxicity assay). Nonetheless, it is likely that blocking such a wide range of MMPs in the presence of TGF-β1 is not advantageous, considering the vast range of actions MMPs are involved in.

Specifically blocking MMP-2 gave seemingly contrasting results. TGF-β1-induced apoptosis was significantly reduced at 4-days; nonetheless the apoptosis was not completely abrogated. These results might suggest that MMP-2 activity is specifically required to contribute to TGF-β1-induced apoptosis in collagen gels. However, why this result was not mirrored by the broad-spectrum inhibitor is not understood. Whether this effect is due to side effects (secondary effects) of the inhibitors which are separate from their role as MMP inhibitors is not known.

Further analysis of MMP and TIMP gene expression levels in normal scar cells by RT-PCR did however reveal a correlation with apoptosis. MMP-1, 2, 3 and -13 were studied, of these only MMP-1 and -2 appeared to be significantly affected by TGF-β1. MMP-1 and -2 were elevated in anchored + TGF-β1 gels, to a similar level to that seen in contractile gels maintained in minimal growth medium alone. When

contractile gels were treated with TGF-β1, MMP-2 was further, significantly, increased.

In parallel with this increasing MMP gene expression in collagen gels + TGF-β1, normal scar cells decreased their expression of TIMP-2. TIMP-2 was expressed at its highest in anchored collagen gels maintained in minimal growth medium alone. TIMP-2 levels were significantly reduced in collagen gels treated with TGF-β1, but not significantly in untreated contractile gels. Normal scar cells therefore show a correlation of increased MMP expression and decreased TIMP expression with TGF-β1-induced fibroblast apoptosis, but only increased gene expression levels of MMPs are associated with collagen contraction-induced apoptosis.

Intriguingly, keloid scar cells show no such correlations. The presence of TGF-β1, despite successfully inducing apoptosis, has no significant effect on either MMP or TIMP expression in anchored collagen gels. This is surprising given the correlations identified with normal scar cells and induction of apoptosis. In addition, keloid scar cells do express MMPs in contractile collagen gels maintained in minimal growth medium (equivalent to that seen with normal scar cells), however there is an absence of apoptosis. These results lead to the conclusion that the gene expression levels of these ECM-degrading enzymes may not correlate directly with apoptosis induction in keloid scar cells.

The effect of TGF-β1 on the expression of MMP genes has been reported previously by others. Uria *et al.* (1998) studied the effects of TGF-β1 on the expression on MMP-1 and MMP-13 in immortalised human KMS-6 embryonic fibroblasts and found TGF-β1 to strongly induce collagenase-3 (MMP-13) expression; in contrast however they found TGF-β1 to down-regulate the expression of MMP-1. Uria *et al's* (1998) study involved Northern blot analysis with specific cDNA probes, cells were initially cultured on monolayer 100mm dishes in SFM before being exposed to various growth factors; in contrast, the results presented in this chapter are from scar fibroblasts cultured in 3-D collagen gels. It is possible that TGF-β1 may affect MMP secretion differently in 3-D collagen culture. Indeed, Ravanti *et al.* (1999b) found that MMP-13 is only expressed by human skin fibroblasts when in contact with 3-D

collagen, however this was found to be down-regulated by TGF-β1; cells were cultured in 1% FCS similar to the assays carried out in this chapter. In another study with human gingival fibroblasts, Ravanti *et al.* (1999a) found that the expression of MMP-13 and MMP-3 were significantly increased in monolayer cultures and in 3-D collagen gels with the addition of TGF-β1. Ravanti *et al's* studies involved RT-PCR analysis, Northern blot and western blot analysis. These two studies by Ravanti *et al.* clearly show a fundamental difference between human dermal fibroblasts and gingival fibroblasts with respect to their collagenase activity; it is feasible that scar fibroblasts also display inherent differences in their collagenase activity to that of dermal fibroblasts, and most likely keloid scar versus normal scar too. Nonetheless, TGF-β1 clearly has an important role in regulating the expression of these enzymes.

Results presented in Figure 6.12 showing activation of the MMP-2 enzyme by zymographic analysis of cell conditioned media after collagen gel culture confirmed the MMP/TIMP expression results for normal scar fibroblasts. Findings demonstrated that collagen contraction alone or anchored collagen gel culture in the presence of TGF-β1 lead to a significant increase in active MMP-2 protein levels for normal scar-derived fibroblasts. However, results from keloid scar cells were again confusing, showing that MMP-2 activation was significantly upregulated during collagen contraction although no apoptosis occurred. In contrast, TGF-β1 did not cause a significant increase in MMP-2 activity, although it clearly induced equivalent apoptosis to that seen during collagen gel contraction by normal scar cells. This result demonstrates that collagen contraction-induced apoptosis and TGF-β1-induced apoptosis do not solely rely on the induction of increased MMP activity in keloid scar cell-seeded collagen gels. There maybe another essential element to the biochemical apoptosis cues which keloid scar cells fail to express or respond too.

6.3.3 Are Biochemical Cues of Apoptosis Produced during TGF-β1-Induced Apoptosis?

Silver staining of the protein preparations from TGF-β1-treated cell-conditioned anchored collagen matrices revealed an increase in polypeptides of <50kDa, over that seen in untreated gels. The molecular weight pattern of these was broadly similar to that seen in contractile collagen matrices. This result was obtained with both normal

scar and keloid scar cell conditioned collagen matrices. In addition, homogenised gel preparations from normal scar and keloid scar cell-conditioned anchored matrices that had been treated with TGF-β1, were able to induce cell death of monolayer cultured fibroblasts (normal scar and keloid scar-derived). This induction of apoptosis in monolayer cells is not simply due to TGF-β1 alone since results in Chapter 5 (Figure 5.19) demonstrate that TGF-β1 itself does not induce apoptosis under these conditions.

Taken together, these results demonstrate that both normal scar and keloid scar cells are able to condition anchored collagen gels in the presence of TGF-β1, in such a way as to produce biochemical cues of apoptosis (as demonstrated in Chapter 4 with normal scar cell-conditioned contractile matrices).

Given the apparent fault in collagen contraction driven apoptosis demonstrated by keloid scar fibroblasts, it is perhaps surprising that TGF-β1 induces apoptosis in 3-D collagen, to an equivalent degree for both keloid scar and normal scar fibroblasts. It is feasible that keloid scar cells have a fault in their autocrine production/activation or bioavailability of TGF-β1. The addition of exogenous TGF-β1 may bypass this fault, by allowing cells to differentiate into activated myofibroblasts (shown previously in Chapter 5), allowing cells to sufficiently condition the collagen gel to produce biochemical cues of apoptosis or by allowing cells to respond to biochemical cues of apoptosis.

6.3.4 What Determines the Capability of Scar Fibroblasts to Respond to Matrix-Related Apoptosis Cues?

The fact that keloid scar cells are able to respond to the normal scar cell-conditioned contractile collagen gel homogenates but not pure products of matrix breakdown, suggests that the cell-conditioned solution contains other elements that are required for successful apoptosis. Whether these are required alone or in addition to collagen breakdown products is unknown. In addition, keloid scar cells are able to respond to normal scar and keloid scar cell-conditioned anchored collagen gel homogenates that have been treated with TGF-β1. This would suggest that TGF-β1 is possibly required either directly or indirectly to facilitate apoptosis in collagen gels. Hypothetically, it is

possible that the production of apoptosis cues by normal scar cells during the conditioning of collagen gels involves autocrine TGF-β1. TGF-β1 levels may build up during the period the matrix is retained anchored; active TGF-β1 may even help mediate the remodelling of the matrix during the period the gel contracts through activation of MMPs. Alternatively, MMPs are known to play a role in TGF-β1 activation (Yu and Stamenkovic, 2000; Dallas *et al.*, 2002). TGF-β1's effects on apoptosis may not be simply through its action on MMP activity, judging by the inability of keloid scar cells to respond to pure products of extracellular matrix remodelling. In this manner, TGF-β1 may be able to control extracellular matrix degradation as well as being distinctly involved in the cellular response to biochemical breakdown products.

In respect of this, experiments were carried out to test the effect the presence of TGF-β1 has on collagenase digested collagen gel fragments and synthetic RGD-peptide treatment of keloid scar cell monolayer cultures. Surprisingly, TGF-β1 enabled the keloid scar cell monolayers to respond to both forms of peptide solutions, undergoing significant levels of apoptosis. Furthermore, in agreement with the findings in section 6.2.1, the apoptotic response was not dictated by the level of myofibroblast differentiation present. It is clear that for keloid scar cells at least that TGF-β1 exhibits a role in determining a cells potential to undergo matrix-related apoptosis that is distinct from its action on MMP expression and myofibroblast differentiation. It is feasible that normal scar cell monolayers are able to respond to these fragments in the absence of exogenous TGF-β1 (presented in Chapter 4) due to higher autocrine expression/activation or bioavailability of TGF-β1 in monolayer culture. In order to determine this, scar cells could to be assessed for their secretion and activation levels of TGF-β1 alongside attempting to induce RGD-peptide apoptosis of normal scar cells in the presence of TGF-β1 blocking agents.

6.3.5 Similarities between TGF-β1-induced Apoptosis and Collagen Contraction-Induced Apoptosis.

In addition to TGF-β1-induced apoptosis and collagen contraction-induced apoptosis both being specific to a 3-D collagen matrix, the results presented in this chapter also demonstrate other important similarities. Results investigating the involvement of

caspase-3 in TGF-β1-induced apoptosis and collagen contraction-induced apoptosis found that this enzyme is centrally involved in the cellular response to both apoptotic processes. Furthermore, on studying the degree of contraction induced by normal scar cells in response to TGF-β1 and TGF-β1-blocking agents, results determined that the extent of gel contraction does not specifically correlate with the amount of apoptosis occurring within the matrix. This was also found to be true of collagen contraction-induced apoptosis, previously presented in Chapter 3. Where keloid scar cells are capable of contracting a collagen matrix over 7-days, to an equivalent degree as that seen with normal scar cells, yet no apoptosis was detected in keloid scar cell-seeded matrices.

6.3.6 Summary

This chapter demonstrates important parallels between the apoptotic induction of fibroblasts by addition of exogenous TGF-β1 to collagen gels and that produced by collagen contraction. This may indicate that TGF-β1 plays an important role in collagen contraction-induced apoptosis and certainly in the apparent defect demonstrated by keloid scar cells. Its role is not solely via the induction of the myofibroblast phenotype or MMP activation but appears more distinct in the ability of at least pathological cells to respond to the apoptotic cues. In addition, this chapter also demonstrates that the fault with keloid scar cells is not simply an inability of cells to breakdown the extracellular matrix, but rather a failure to produce the correct signalling environment to allow effective extracellular matrix degradation and cellular response.

Chapter 7

Discussion of Research

Discussion

The work carried out in this thesis has been aimed at investigating the apoptosis cues that takes place during the end phase of tissue repair. Successful apoptosis leads to the clearance of redundant wound cells and cessation of the wound repair process. This event may not occur in pathological scar conditions and may explain the prolonged presence of activated fibroblasts. To date the mechanisms and triggers that signal cells to undergo apoptosis in the later stages of wound healing are unknown. Work carried out by Fluck *et al.* (1998) and Buckley *et al.* (1999) providing clues for the role of extracellular matrix proteins and their potential breakdown products in apoptosis induction formed the foundation of the work carried out in this thesis. The wound healing apoptosis models employed by these research groups were thus used to dissect the specific mechanisms involved in the induction of apoptosis. In addition, the investigation of pathological defects was used to further enhance these findings.

7.1 Summation of the Major Findings of this Thesis

Investigations utilising a contractile collagen *in vitro* model of wound healing found that normal scar fibroblasts (like dermal fibroblasts, Fluck *et al.*, 1998), but not keloid scar fibroblasts underwent apoptosis during the contraction of 3-D collagen but not fibrin gels. The defect displayed by keloid scar cells was specific to that induced in this wound model, since these cells were equivalent to normal scar cells in their sensitivity to chemical methods of apoptosis induction, as judged by cell viability assays, DNA nick-end labelling, stabilisation of p53, and activation of caspase-3 and PARP. Furthermore, this defect was not simply due to any absence or reduction in collagen contraction by keloid scar-derived cells.

Whilst there is undoubtedly a mechanical component to the mechanisms of collagen contraction-induced apoptosis, elimination of cell-tension, cell-cell and cell-matrix contacts by use of cytochalasin disruption of actin cytoskeleton or via classical anoikis assays, was found insufficient to cause apoptosis over the same time course used for collagen contraction-induced apoptosis. The mechanism of collagen contraction-induced apoptosis was determined to involve biochemical cues of apoptosis, since homogenates of collagen gels conditioned and contracted by normal

scar cells were found able to induce apoptosis of both normal scar and keloid scar cells in monolayer, whereas gels contracted by keloid scar cells could not.

Investigations into the identity of these cues initially centred on matrix remodelling since collagenase activity is one of the major biochemical differences between anchored and contractile collagen gels. In addition, Buckley *et al.* (1999) determined that small potential breakdown products of matrix remodelling (RGD-motif containing peptides) could directly induce apoptosis. It was therefore hypothesised that the main biochemical cues produced during the contraction of collagen were remodelling-induced breakdown products that included small soluble RGD-motif-containing matrix protein fragments. The activity of the major perpetrators of matrix remodelling (the MMPs) were found to be directly implicated in collagen contraction-induced apoptosis through blocking experiments using a broad-spectrum MMP inhibitor (Ilomastat, blocking MMP-1, 2, 3, 8 and 9), a specific inhibitor of MMP-2 and a physiological inhibitor (recombinant TIMP-2). However, MMP inhibition had no obvious effect on actual gel contraction. These findings were backed-up by results demonstrating a decrease in polypeptides of 150-50kDa together with a concomitant increase in small polypeptides (<50kDa) in protein preparations from collagen gels that had been contracted by normal scar cells; suggestive of collagen gel degradation. In contrast, keloid scar cell-contracted gels did not, exhibiting a similar pattern of polypeptides to that seen in anchored collagen gels containing either cell type. Further circumstantial support came from analysis of the gene expression and activation levels of MMPs and associated proteins. Collagen contraction by normal scar cells was associated with significantly higher gene expression of MMP-1, -2, -3 and –13, but no change in TIMPs, along with an increase in activation of MMP-2. However, the defect displayed by keloid scar cells to contraction-induced apoptosis does not appear to be simply due to a reduction in MMP activity of these cells, since they too show significantly increased MMP expression and activity on contraction, and to equivalent levels to normal scar cells. Surprisingly, it was determined that keloid scar cells could not respond to pro-apoptotic breakdown products of collagen/matrix remodelling, since unlike normal scar cells, treatment of monolayer cultures of keloid scar cells with breakdown products of collagenase D-digestion of acellular collagen gels or pure synthetic RGD peptides did not result in apoptosis.

Studies examining the role of myofibroblast differentiation in the ability of cells to undergo apoptosis in response to collagen contraction determined that the presence of the myofibroblast phenotype certainly correlates with successful apoptosis, with normal scar but not keloid scar cells, differentiating into myofibroblasts in collagen gels. However, the presence of the myofibroblast phenotype alone in collagen gels did not result in apoptosis. The addition of active TGF-β1 in a successful attempt to induce myofibroblast differentiation of keloid scar cells in collagen gels was intriguingly also found to induce apoptosis of both normal scar and keloid scar cells in anchored collagen but not fibrin gels, and to a level equivalent to that normally seen in contractile collagen gels containing normal scar cells.

Further investigation of the mechanisms involved in TGF-β1-induced apoptosis revealed that, like collagen contraction-induced apoptosis, caspase-3 activation was centrally involved (as determined by specific inhibition during collagen gel culture). Furthermore, TGF-β1-induced apoptosis is dependent on the 3-D nature of the gels and not merely a collagenous substrate. Due to the similarities between the prerequisites displayed by both TGF-β1-induced and collagen contraction-induced apoptosis a link between the two was proposed. TGF-β1 was found to be absolutely required for collagen contraction-induced apoptosis, as determined by blocking experiments using mannose-6-phosphate as an inhibitor of latent TGF-β1 activation or a TGF-β1-blocking antibody. Furthermore, the role of TGF-β1 appeared to be distinct from its affect on myofibroblast differentiation, since delaying the timing of TGF-β1 blockade still allowed overt myofibroblast differentiation (as judged by α-SMA expression) and yet blocked collagen contraction-induced apoptosis.

Investigations assessing the involvement of MMPs in TGF-β1-induced apoptosis by blocking a broad spectrum of MMPs (Ilomastat) or MMP-2 alone produced contrasting and inconclusive results. Nevertheless, a correlation was again found between MMP activity and induction of apoptosis for normal scar cells, where TGF-β1 induced significant increases in MMP-1 and -2 gene expression, with a concomitant reduction in TIMP-2 expression and an increase in MMP-2 activation. Once more however, keloid scar cells showed no such correlation, with TGF-β1 having no significant effect on gene expression or activity of the MMPs and TIMPS

studied, despite successfully inducing apoptosis of these cells. Despite this, TGF-β1 treatment of keloid scar cell-seeded anchored collagen gels, enables the cells to condition the gel in such a way as to produce small polypeptides of <50kDa, similar to those produced in normal scar cell contracted collagen gels. Moreover, TGF-β1 treatment allows keloid scar cells to condition collagen gels so as to produce cues of apoptosis that are capable of inducing monolayer cultures of scar fibroblasts to undergo apoptosis. Finally, the addition of exogenous TGF-β1 to monolayer cultures of keloid scar cells enabled these cells to undergo apoptosis in response to collagenase digested acellular collagen fragments or synthetic RGD-motif-containing peptides.

7.2 Rationale for The Use of Normal Scar Cells as an Appropriate Control to Keloid Scar Cells

As previously discussed in section 1.8, normal scar-derived cells were chosen as an appropriate control for comparison with those derived from the pathological condition 'keloid scarring'. The reason for using normal scar-derived cells rather than dermal fibroblasts was due to the fact that, like keloid scar cells normal scar cells had been subjected to the process of wound healing; this process may potentially irretrievably change the phenotype of the cell. In addition, the disposition of dermal fibroblasts towards normal or pathological scarring is unknown since they are derived from previously unwounded tissues. For these reasons, using dermal fibroblasts in a wound-healing model as a control to be compared with pathological scar cells may not produce meaningful, reliable or consistent results. Normal scar cells instead, have realised their fate as 'normal' and are thus a more appropriate control for keloid scar-derived cells.

The investigations carried out within this thesis should ideally have used normal scar and keloid scar fibroblasts derived from equivalent body-sites and age-matched patients. Although age matching was achieved, matching of body sites was particularly difficult as the elective surgical procedures performed at Mount Vernon Hospital throughout this study involved normal scar and keloid scar removal mainly from consistently different body sites (see Appendix I). Most keloid scars were from the ears, and normal scars are rarely sufficiently problematic on ears to warrant surgical resection. The fact that scar cells from these two scar types have been derived

from mainly different body sites, although there was some overlap, may be seen as a potential problem as there could be inter-body site differences in cell behaviour. However, the fact that the apoptosis results were so clearly different between normal and pathological cells and consistently exhibited by each cell strain for that scar type argues against any influence of the different body sites. Nevertheless, it is acknowledged that ideally site matched cell strains should be compared.

7.3 Is the Mechanism of Wound Healing-Induced Apoptosis Solely Mechanical?

Results from experiments with Cytochalasin D and anoikis induction in Chapter 4, revealed that simply removing cell-cell and cell-matrix contacts, cell-tension, serum and even extracellular matrix proteins were insufficient to signal fibroblasts to undergo apoptosis, over the same time-course used for collagen contraction-induced apoptosis. It was concluded therefore that the apoptosis induced during collagen contraction *in vitro* (late granulation tissue remodelling – *in vivo*) was not through loss of cell-tension and cellular contacts alone.

Results presented in this thesis clearly and repeatedly demonstrate that collagen matrix contraction does not correlate with fibroblast apoptosis; as such the degree of matrix contraction cannot be used as an accurate measure of apoptosis as previously suggested (Grinnell *et al.*, 1999; Zhu *et al.*, 2001). Nevertheless, examination of the rate of contraction by keloid scar versus normal scar cells identified an interesting albeit small difference in the initial spurt of contraction (up to 2-days), being significantly slower for keloid scar cells. This may indicate a difference in the mechanics of collagen contraction by normal scar and keloid scar cells, which of course may influence a cells ability to produce/ respond to apoptotic cues. This might be further clarified by examining the rate of collagen gel contraction in the presence of various treatments determined in this thesis to have an affect on collagen contraction-induced apoptosis.

Undoubtedly, the removal of cell-tension (stress-relaxation) is somehow involved in the initiation of events that culminate in the apoptosis that occurs during collagen contraction. It is feasible, that rather than directly signalling fibroblasts to undergo apoptosis; the removal of cell-tension may act as a trigger, initiating secondary events that signal apoptosis. Conceivably, distinct cell-integrins may act to transduce the

mechanical stimulus into the cell, activating biochemical changes, which in turn induces apoptosis. Evidence for this is provided by the ability of normal scar cells to condition collagen gels during contraction in such a way as to be able to signal monolayer cultures of scar fibroblasts to undergo apoptosis (Chapter 4).

The aetiology of keloid scars may potentially stem from an absence of the resolution phase of wound healing due to the removal of cell-tension never taking place. Theoretically, removal of wound-tension usually occurs *in vivo* once the leading edges of migrating keratinocytes have come together and formed the neoepidermis. The dissipation of wound-tension potentially leads to decreased cell activation and survival signals (as occurs during stress-relaxation *in vitro*). The over-active nature of keloid scars from the prolonged expression of survival signals and cell activation, suggests that wound-tension may fail to be eliminated, leading to aberrant wound resolution. Indeed, keloid scar-derived fibroblasts in contractile collagen gels appear very taut and active in comparison to normal scar-derived fibroblasts, which appear loose, withered and relaxed (Figure 3.6). Hypothetically, this could be through increased cross-linking of the extracellular matrix, which may play a role in the failure of cells to undergo apoptosis to wound-healing cues, simply through increased rigidity or an inability to be broken-down to produce specific extracellular matrix fragments capable of inducing apoptosis. Levels of fibronectin, collagens and transglutaminase have been found to be over-expressed in pathological scars (Babu et al., 1989; Di Cesare et al., 1990; Linge *et al.*, 2005). Results presented by Linge *et al.* (2005) discovered that the over-expression of tissue transglutaminase by hypertrophic scar cells played a significant role in preventing these cells from responding to the apoptotic effects of collagen gel contraction whilst in low serum containing media. At present however, no studies have been carried out to assess the level of expression of tissue transglutaminase by keloid scar cells, this could be further investigated by *in situ* staining of cell-conditioned contractile collagen gels, comparing the level of expression of tissue transglutaminase in normal scar versus keloid scar cell-seeded matrices. In addition, SDS-PAGE and western blotting could be carried out with cell-conditioned contractile collagen gels cell-lysates, comparing the level of tissue transglutaminase (and indeed other cross-linking proteins) expressed in keloid scar cell-seeded gels to that of normal dermis- and normal scar cell-seeded gels.

Keloid scars have also been reported to exhibit reduced degradation of extracellular matrix proteins, suggested to be caused by reduced collagenase activity (Abergel *et al.*, 1985; Ghahary *et al.*, 1996), potentially through an accumulation of α2-macroglobulin and α1-antitrypsin (collagenase inhibitors) detected in keloid scars (Diegelmann *et al.*, 1977; Ueyama *et al.*, 1992).

7.4 The Involvement of Collagen Remodelling Components in Collagen Contraction-Induced Apoptosis

Having established the existence of biochemical cues of apoptosis, attempts to define them initially concentrated on a previously under-examined hypothesis; that products of collagen remodelling induce cellular apoptosis in contractile collagen gels. The fact that collagenase activity is upregulated during collagen contraction supports this theory and thus warranted further investigation.

7.4.1 Matrix Metalloproteinase Expression in Normal Scar Cells

There is some evidence, which suggests that within late granulation tissue, the synthesis of extracellular matrix proteins decreases in parallel with increased synthesis of collagenases (Unemore and Werb, 1986; Klein *et al.*, 1991; Grinnell, 1994). Therefore, during this phase of wound healing the extracellular matrix is being progressively broken-down. In this manner, cells are probably in an environment containing increasing amounts of extracellular matrix fragments (small polypeptides), species of which are known to induce fibroblast apoptosis (Buckley *et al.*, 1999; Perlot *et al.*, 2002).

Work carried out in this thesis, using a variety of MMP inhibitors in the 3-D collagen gel model of wound healing has clearly demonstrated an absolute requirement for MMP action in the induction of apoptosis during collagen contraction by normal scar cells. This is supported by an increase in MMP gene expression and MMP-2 activity seen during contraction, along with the apparent increase in matrix breakdown products, demonstrated by SDS-PAGE (Chapter 4).

Both the absence of MMP gene expression and presence of TIMP-2 expressed by normal scar cells maintained in anchored collagen matrices may explain the lack of

apoptosis within these gels; through deficient extracellular matrix degradation, again demonstrated by SDS-PAGE analysis (Section 4.2.2).

Ideally further quantitative experiments to investigate the expression and involvement of a more extensive range of MMPs and TIMPs should be carried out using; quantitative RT-PCR analysis, immunoblotting, ELISA, reverse zymography and siRNA methods. Whether MMP involvement is simply through production of extracellular matrix fragments or via other means is still unclear. A better idea of the link between MMP action and apoptosis might be gained by attempting to isolate and define molecular species capable of inducing apoptosis from gels contracted by normal scar cells. This could be achieved by size fractionation of protein fragments from normal scar versus keloid scar cell-conditioned contractile collagen gels and isolation of the elements capable of inducing apoptosis or by HPLC (high performance liquid chromatography) analysis. HPLC analysis would provide an accurate analysis of the protein profiles of normal scar and keloid scar cell-conditioned contractile collagen gel samples. If any differences in the protein expression profile is detected between normal scar and keloid scar cell-conditioned contractile collagen gel samples, these distinct proteins could be isolated and sequenced. Further characterisation could involve western blotting for specific peptides thought to be involved fibroblast apoptosis (for example, antibodies recognising peptides with an RGD-motif).

7.4.2 A Possible Role for ECM Derived Polypeptides in Apoptosis of Normal Scar Cells

Although it remains to be confirmed that during collagen gel contraction normal scar cells remodel the extracellular matrix producing small extracellular matrix fragments, which are capable of inducing fibroblast apoptosis (see hypothetical model Figure 7.1); there is at least circumstantial evidence for this theory. Investigations found that homogenised contractile-collagen gels conditioned by normal scar cells contained an increased amount of polypeptides of <50kDa; furthermore, this homogenised solution signalled scar fibroblast monolayer cultures to undergo cell death (section 4.2.1).

Interestingly, alongside the increase in polypeptide bands of <50kDa, there is a decrease of polypeptide bands between 100 and 150kDa; the correct size for α-chains

of collagen (Linge *et al.*, 2005). Analysis by western blotting or HPLC methods would confirm this. If this is the case, it would suggest that the increase in polypeptides of <50kDa are products of collagen cleavage.

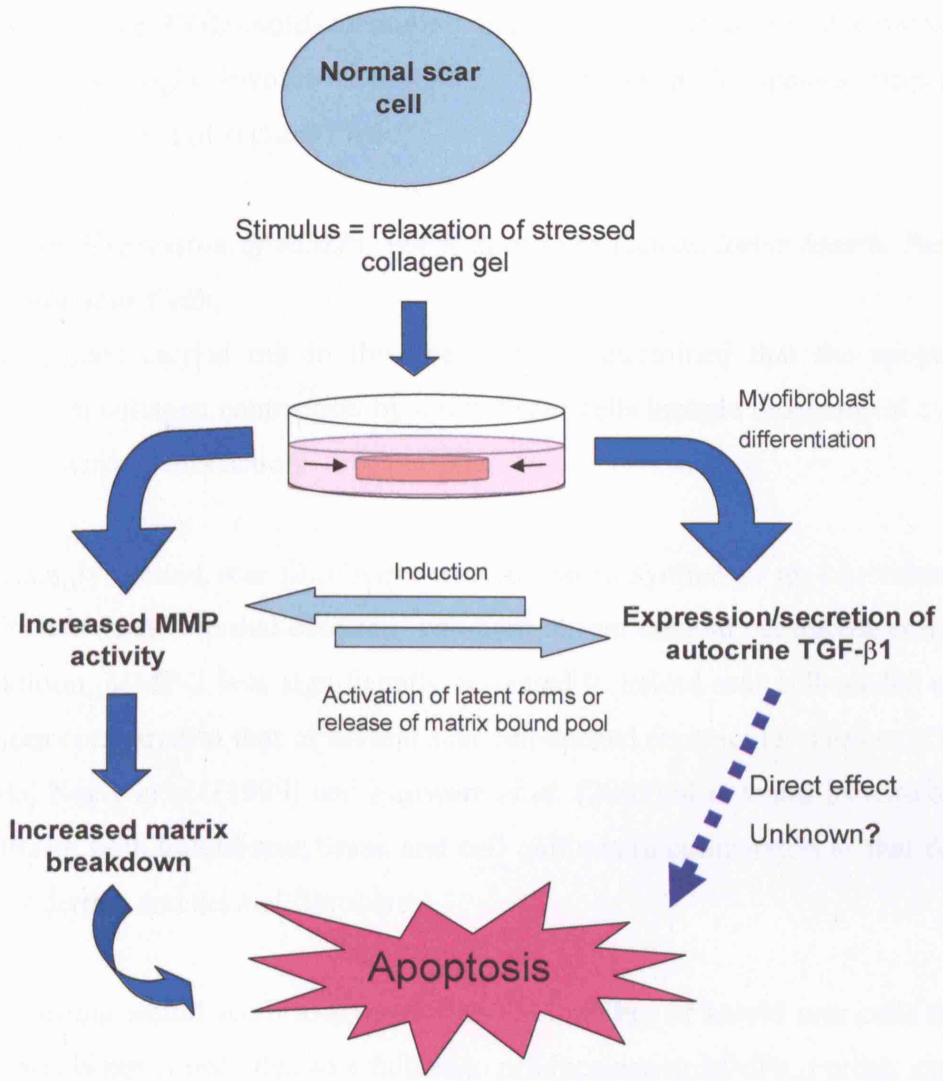


Figure 7.1 Hypothetical model of the events that take place during normal scar cell contraction of pre-stressed collagen matrices in 1% NGM. Specifically, normal scar cells signal the activation of MMPs, which leads to increased breakdown of the extracellular matrix alongside increased activation of autocrine TGF-β1, from increased secretion or release from matrix bound pool, followed by activation.

It is clear that products of collagen digestion can induce apoptosis since a solution of collagenase-digested acellular collagen gels containing multiple extracellular matrix fragments of various sizes was able to induce normal scar-derived monolayer fibroblast cultures to undergo apoptosis; even though they do not undergo anoikis

over the same time-course (section 4.2.1.1). In addition, a solution of synthetic RGD-motif-containing peptides also specifically induced normal scar monolayer fibroblast cultures to undergo apoptosis under conditions where attachment was RGD-independent (section 4.2.3). Whether normal scar cells remodel a contractile collagen gel to produce RGD-motif-containing peptides remains to be elucidated. Further experiments might involve determining the polypeptide species responsible as detailed at the end of section 7.4.1.

7.4.3 The Expression of MMPs and Response to Extracellular Matrix Polypeptides by Keloid Scar Cells

Investigations carried out in this thesis have determined that the apoptosis cues involved in collagen contraction by normal scar cells include biochemical events such as MMP activity and action.

Surprisingly, keloid scar fibroblasts were found to synthesise an equivalent level of MMPs and TIMPs to that detected with normal scar cells in contractile collagen gels. In addition, MMP-2 was significantly activated in keloid scar cell-seeded contractile matrices compared to that of normal scar cell-seeded contractile matrices (Chapter 4). Indeed, Neely *et al.* (1999) and Fujiwara *et al.* (2005) also found increased levels of MMP-2 in both keloid scar tissue and cell cultures in comparison to that detected in normal dermis and dermal fibroblasts.

These results would seem to suggest that the inability of keloid scar cells to undergo apoptosis is not simply due to a failure to produce active MMPs. Further experiments to investigate the protein expression and activation of a broader range of MMPs and TIMPs might prove conclusive in this respect, this could include: quantitative RT-PCR analysis, immunoblotting or ELISA for the activity of various MMPs and TIMPs, and reverse zymography comparing samples derived from normal scar- and keloid scar cell-conditioned contractile-collagen gels. Additionally, functional studies with normal scar-cells could be carried out including siRNA techniques (silencing of specific genes by RNA interference) blocking the transcription of specific MMPs/TIMPs. Any obvious involvement of specific MMPs or TIMPs in the induction of collagen contraction-induced apoptosis could then be further studied in keloid scar cells.

These results suggest that keloid scar cells are able to synthesise and potentially activate extracellular matrix-degrading enzymes at an equivalent level to that of normal scar cells. However, results from SDS-PAGE analysis of the protein-banding pattern of keloid scar cell-conditioned contractile collagen gels revealed that matrix breakdown by these cells appeared insufficient. Moreover, a solution of homogenised keloid scar cell-conditioned contractile collagen gel failed to signal normal scar fibroblasts to undergo apoptosis. Thus, keloid scar cells do not condition a contractile matrix in such a way as to produce biochemical cues of apoptosis, which may be simply extracellular matrix fragments, as judged by SDS-PAGE analysis (section 4.2.2). This possibility may be due to excessive cross-linking and stabilisation of the matrix by the keloid cells, which could be examined as detailed at the end of section 7.3. However, the simple inability to produce extracellular matrix breakdown products that are cues of apoptosis does not seem to be the sole component of the defect exhibited by keloid scar cells.

As well as not producing small extracellular matrix fragments, keloid scar fibroblast monolayer cultures were also unable to respond to a solution of collagenase digested acellular collagen gel fragments or synthetic RGD-motif containing peptides (section 4.2.3). Intriguingly however, keloid scar cell monolayer cultures were able to respond to a solution of homogenised normal scar cell-conditioned contractile collagen gel. These results suggest that an additional element, potentially a permissive co-factor either produced by the normal scar cells or a serum-derived factor remaining present within the collagen gels is required to normalise the apoptotic response of keloid scar cells (Figure 7.2 summarises the faults demonstrated by keloid scar cells). It is possible that this co-factor might also be essential for the normal cellular response to collagen contraction or alternatively is a distinct requirement of abnormal cells to function normally. Further studies might involve HPLC analysis of the products from normal scar versus keloid scar cell-remodelled collagen gels, either of the conditioned medium or the gel itself. Molecular species present within normal scar cell-conditioned collagen gels and not present in keloid scar cell-conditioned collagen gels could then be isolated and characterised.

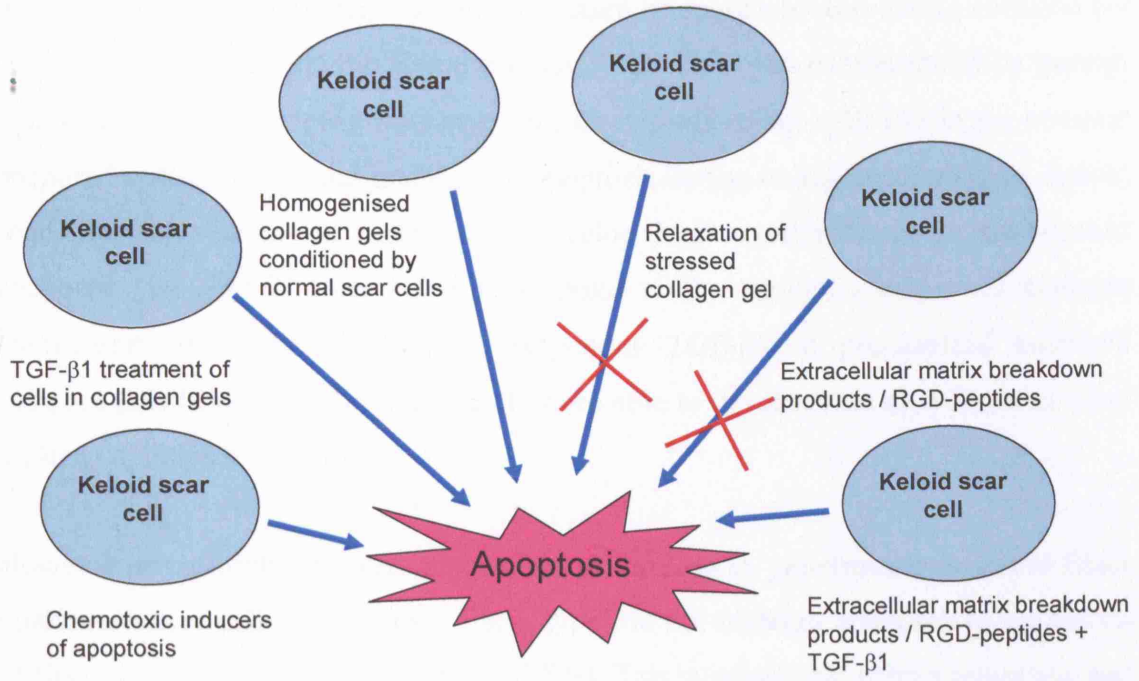


Figure 7.2 Illustrated summary of what is known about the response of keloid scar cells to various apoptosis induction cues. This involves potential wound healing apoptosis cues and chemical apoptosis cues.

7.5 Is the Myofibroblast Phenotype Required for Cells to Respond to Collagen Contraction-Induced Apoptosis?

It has been suggested by Hinz *et al.* (2000) that the myofibroblast is the main cell type present in granulation tissue and is instrumental in wound contraction. During granulation tissue remodelling the myofibroblast is proposed to be the primary cell-type responsible for establishing contacts with the extracellular matrix; thus providing a network, which allows tractional forces to be transmitted across the wound-bed and into the cells (Welch *et al.*, 1990; Singer *et al.*, 1984). A number of studies have postulated that it is specifically myofibroblasts that undergo apoptosis to the specific cues of granulation tissue remodelling (Darby *et al.*, 1990; Clark, 1993; Desmouliere *et al.*, 1993).

Results presented in this thesis demonstrate that the presence of myofibroblasts in collagen gels does indeed correlate with the ability of cells to undergo apoptosis (Chapter 5). Specifically, the ability to develop into myofibroblasts during the pre-stressed anchored period before the release of collagen gels (thought to mimic early

granulation tissue) correlates with the induction of apoptosis seen during collagen gel contraction (late granulation tissue remodelling). This was demonstrated by normal scar fibroblasts developing into myofibroblasts (expressing α-SMA) in pre-stressed anchored collagen gels and undergoing apoptosis during collagen contraction. Keloid scar fibroblasts conversely, failed to develop into myofibroblasts in pre-stressed anchored gels and in turn failed to respond to the apoptotic effects of collagen contraction. With the addition of exogenous TGF-β1 to pre-stressed anchored collagen gels however, keloid scar cells were able to develop into myofibroblasts and undergo apoptosis (Section 5.2.4).

However, myofibroblasts were also found to develop in pre-stressed anchored fibrin matrices, where cells (regardless of scar type) do not undergo apoptosis to the effects of fibrin gel contraction (Figure 5.6 and 5.9). This suggests that matrix relaxation and the myofibroblast phenotype together are insufficient for apoptosis and further, that a collagenous environment is specifically required. This may be due to distinct integrins being expressed by myofibroblasts that may act to alter the function of the cell with extracellular matrix environment. Thus, only collagen integrins (α1β1 and α2β1) may play a role in collagen contraction-induced apoptosis, rather than fibrin integrins, such as αvβ3. Indeed, Niland *et al.* (2001) found that blocking α1β1 and α2β1 integrin binding inhibited apoptosis of normal dermal fibroblasts during collagen contraction, however as previously mentioned these integrins also affect MMP activity (Seltzer *et al.*, 1994; Langholtz *et al.*, 1995) amongst a plethora of other bioactivities (as previously discussed in section 4.1.3). Further experiments comparing normal scar cells with keloid scar cells to define the integrin expression profile and function during collagen contraction might include: immunoblotting of lysed day-7 cell-conditioned contractile collagen gels, protein microarrays or *in situ* immunohistochemistry of day-7 cell-conditioned collagen gels for specific integrins (α1β1 and α2β1) known to be involved in collagen contraction, siRNA functional studies and quantitative RT-PCR to assess the mRNA expression levels of integrins, namely α1β1 and α2β1.

This thesis has however, produced evidence suggesting that the presence of the myofibroblast phenotype is not required for apoptosis induction by products of

extracellular matrix remodelling, since they were not present during induction of apoptosis by synthetic RGD-peptides (Figure 6.19). These latter results indicate either that the myofibroblast phenotype is required to produce the apoptotic cues rather than respond to them, or alternatively that it is simply not required at all. The possibility that myofibroblasts are not required is supported by the work of Kobayashi *et al* (2005), who found that factors such as PDGF that induce myofibroblast differentiation do not affect the apoptosis seen in collagen gels (anchored or contractile).

7.6 Does TGF-β1 have a Distinct Role in Collagen Contraction-Induced Apoptosis?

Results from the inhibition of endogenous TGF-β1 from T0 of normal scar cell-seeded collagen gel culture was found to prevent myofibroblast differentiation as well as the apoptosis induction that occurs on collagen contraction (Section 6.2.1). The interpretation of these initial results could be that myofibroblasts alone are critical for collagen contraction-induced apoptosis. However, when TGF-β1 was inhibited at day 4 of collagen gel culture (concurrent with releasing collagen gels to allow contraction to occur), myofibroblast differentiation did take place, yet collagen contraction-induced apoptosis was again inhibited. The latter results therefore, determined that myofibroblast differentiation alone in a contracting collagen gel is not sufficient for successful apoptosis induction and that TGF-β1 would appear to play a distinct role in collagen contraction-induced apoptosis. Furthermore, the presence of myofibroblasts may simply be indicative that the correct conditions are present for the successful induction of collagen contraction-induced apoptosis.

In light of previous results suggesting the involvement of a permissive co-factor in the cellular response to collagen contraction, TGF-β1 may potentially be acting as this co-factor. For conclusive evidence of this theory, an assay could be carried out to assess the levels of total, active, free and bound TGF-β1 present in anchored and contractile collagen gels containing fibroblasts from either scar type, possibly using a Luciferase bioassay (methods for measuring TGF-β1 levels are extensively reviewed by Jurukovski *et al.*, 2005).

The effect of TGF-β1 on fibroblasts/myofibroblasts to date has usually been to inhibit apoptosis (Zhang *et al.*, 1999; Chen *et al.*, 2003). These *in vitro* experiments however, have evaluated fibroblasts in monolayer culture. The culture of fibroblasts in 3-D collagen matrices is thought to more closely resemble the signalling that occurs during wound repair. As shown throughout this thesis, TGF-β1 is able to signal markedly different responses from fibroblasts depending on the extracellular matrix substrate they are in contact with and indeed whether cultured in 2-D (Figure 5.19) or 3-D conditions (5.15). The finding that TGF-β1 is able to induce apoptosis of fibroblasts in 3-D collagen is therefore not incompatible with contrasting prior publications on cells in monolayer culture.

A role for TGF-β1 in the later stages of wound healing has not so far, been identified. Indeed, it has been previously suggested that the continued presence of TGF-β1 throughout wound repair is somewhat puzzling, given that most of its known actions on fibroblast behaviour are completed within the early stages. The findings presented in this thesis suggest that TGF-β1 may orchestrate the entire wound healing process; where the effect of TGF-β1 evolves with the wound environment. The actions of TGF-β1 being dependant on for instance: the constituents of the extracellular matrix, integrin expression, the growth factors and cytokines available to signal through, the state of cell activation and tension.

7.7 Is the Role of TGF-β1 in Collagen Contraction-Induced Apoptosis Simply via Action on Collagenase Activity?

The addition of exogenous TGF-β1 to the low serum-containing growth media of the wound-healing model signals keloid scar cells to undergo apoptosis not only in contractile collagen gels but also surprisingly in anchored collagen gels. This was mimicked by normal scar cells, where the level of apoptosis induced was equivalent between contractile gels and TGF-β1-treated anchored gels (Figure 5.15). There was no significant difference in the level of apoptosis induced between normal scar and keloid scar cell-seeded anchored gels treated with TGF-β1. This apoptotic effect was specific to that of a 3-D collagenous matrix, similar to matrix contraction-induced apoptosis. In addition, like collagen contraction, caspase-3 is specifically involved in

TGF-β1-induced apoptosis as determined by immunoblotting and the specific inhibition of caspase-3 activation.

7.7.1 Interpretation of TGF-β1's Effect on Normal Scar Cells Cultured in Anchored Collagen Matrices.

On investigating the biochemical cues induced by exogenous TGF-β1 on cells within anchored collagen matrices, it was determined that the apoptosis induced may be through similar cues as collagen contraction-induced apoptosis (the main findings are summarised in Figure 7.3). The gene expression level of both MMP-1 and MMP-2 showed a trend of increasing expression in TGF-β1-treated anchored gels compared to that expressed in anchored gels in the absence of TGF-β1; similar to that expressed in contractile gels (section 6.2.2). Furthermore, MMP-2 protein activation was significantly increased in anchored collagen gels in the presence of TGF-β1 compared to anchored collagen gels in the absence of TGF-β1. The level of MMP-2 activation being equivalent to that expressed in contractile collagen gels. This coincided with a significant decrease in TIMP-2 expression levels in normal scar cell-seeded TGF-β1-treated anchored collagen gels compared to anchored gels in the absence of TGF-β1. There is a similar trend of decreased TIMP-2 expression seen in contractile versus anchored collagen gels in the absence of TGF-β1, although this failed to reach significance.

SDS-PAGE revealed that treatment of cells cultured in anchored collagen gels with TGF-β1 leads to the accumulation of polypeptide fragments of <50kDa mimicking that produced by normal scar cells during the contraction of a collagen matrix. However, surprisingly unlike that seen during collagen contraction, there was no concomitant decrease in the density of bands from 150-50kDa. Moreover, a homogenised solution of normal scar cell-conditioned anchored collagen matrices treated with TGF-β1 signals cell death of both normal scar and keloid scar cell monolayer cultures. These results clearly demonstrate that there are similarities between TGF-β1-induced and collagen contraction-induced apoptosis. It is possible that TGF-β1 simply induces apoptosis in anchored collagen gels through its induction of collagenase activity and thus production of apoptosis-inducing breakdown products (see Figure 7.1). Alternatively, TGF-β1 may act through a distinct mechanism,

although this thesis has produced no clear evidence for this in normal scar cells (discussed further in section 7.9) Whatever the case, it is clear that TGF-β1's action is required for successful collagen contraction-induced apoptosis and that TGF-β1 treatment of cells in collagen gels appears to by-pass the need for a mechanical stimulus.

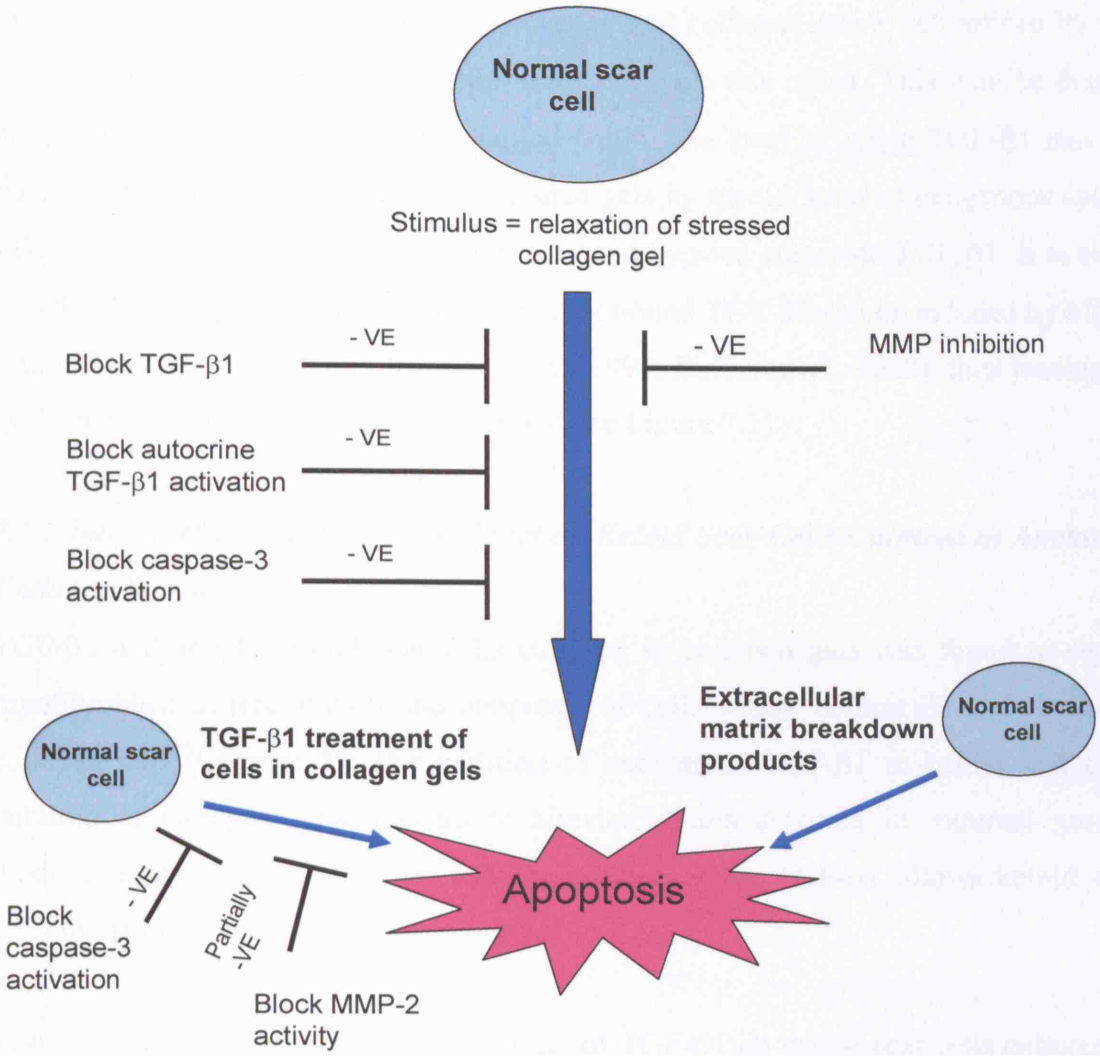


Figure 7.3 Illustrated summary of the similar biochemical events known to be required for normal scar cells to respond to collagen contraction- and TGF-β1-induced apoptosis. Specifically, MMPs, TGF-β1 and caspase-3 are crucial, determined through inhibition assays.

Recently, during the writing of this thesis, a paper was published that also reports TGF-β1-stimulated increase of apoptosis in collagen gels (Kobayashi *et al.*, 2005). This group report that when TGF-β1 was used to treat human fetal lung cells in contractile collagen gels in the absence of serum, a significant increase in apoptosis

was seen. Unlike the findings in this thesis however, they did not see any affect in anchored collagen gels. This may be explained by experimental differences, such as the origin of the cell type used.

It is not fully understood why the addition of exogenous TGF-β1 has this apoptotic effect on fibroblasts within anchored collagen matrices, yet endogenous levels of TGF-β1 (thought to build up in stressed-anchored collagen gels – determined by the presence of α-SMA positive staining) does not have this effect. This may be due to the binding of TGF-β1 into matrix-bound pools. The level of active TGF-β1 may be significantly increased in anchored collagen gels by the addition of exogenous active TGF-β1, compared to the bioavailability of endogenous autocrine TGF-β1. It is clear also that both activation and release of matrix bound TGF-β1 can be induced by MMP action (Uria *et al.*, 1998 and Ravanti *et al.*, 1999; Dallas *et al.*, 2002), thus leading to cyclic activation between the two systems (see Figure 7.1).

7.7.2 Interpretation of TGF-β1's Effect on Keloid Scar Cells Cultured in Anchored Collagen Matrices.

TGF-β1 addition to keloid scar cells cultured in collagen gels was found to signal myofibroblast differentiation and apoptosis of cells within contractile and anchored collagen gels (Chapter 5). The addition of exogenous TGF-β1 to keloid scar cells cultured in collagen gels appears to alleviate faults detected in minimal growth medium alone. Importantly, the addition of exogenous TGF-β1 allows keloid scar cells to act in an equivalent way to normal scar cells.

Further investigations found that the effect of TGF-β1 on keloid scar cells cultured in anchored collagen gels was not through significantly affecting the expression and activation of the MMPs and TIMPs examined. Regardless of this however, SDS-PAGE determined that TGF-β1-treatment of keloid scar cells cultured in anchored collagen gels led to the accumulation of polypeptide fragments of <50kDa, mimicking that seen with TGF-β1 treatment of normal scar cells (Figures 6.13 and 6.14). In addition, a homogenised solution of keloid scar cell-conditioned anchored collagen gels treated with TGF-β1 was capable of inducing apoptosis of monolayer cultures of both normal scar and keloid scar cells (Figure 6.15). It is possible TGF-β1 affects

numerous extracellular matrix-degrading enzymes not studied in this thesis (other than MMPs and TIMPs) and as such a more extensive investigation of these enzymes should to be considered before criticising their involvement and faults in keloid scar cells. Nevertheless, it is clear that TGF-β1 permits keloid scar cells to respond to synthetic RGD-motif containing peptides and the like. In addition therefore, it is possible that all the cues for successful apoptosis are present in these gels and that it is the presence of TGF-β1 itself that allows the cells to respond, or a combination of the two (see hypothetical model Figure 7.4).

To examine whether TGF-β1 has a specific affect on the apoptosis of cell monolayer cultures, an anti-TGF-β1 antibody or M-6-P could be added to the keloid scar cell-conditioned collagen gel homogenates pre-treated with TGF-β1 in order to neutralise any TGF-β1 activity. This would determine whether the degraded collagen fragments produced by keloid scar cells in the presence of TGF-β1 are responsible for the apoptosis alone or whether TGF-β1 is required alongside these fragments to induce monolayer cultured cells to undergo apoptosis.

7.8 Role of TGF-β1 as a Co-factor to Cell Response

It is clear that the exogenous addition of TGF-β1 appears to alleviate many faults detected with keloid scar cells cultured in collagen gels; allowing them to behave in an equivalent way to normal scar fibroblasts. Indeed, this demonstrates that TGF-β1 acts as a permissive co-factor with keloid scar cells (Figure 7.4). Whether TGF-β1 also acts as a co-factor to the normal cellular response to wound-healing apoptosis cues is not determined (Figure 7.1). Results with keloid scar-derived cells provide evidence for a potential fault in the autocrine expression/activation or bioavailability of TGF-β1, shown by aberrant myofibroblast differentiation in pre-stressed anchored collagen gels (Chapter 5). This is implied and is corroborated by the ability of exogenous TGF-β1 to cause keloid scar cells to respond to RGD-peptides and collagenase digested collagen gel fragments (Figure 6.17 and 6.18), thus indicating that autocrine TGF-β1 is insufficient in these cells. In this manner, keloid scar cells may be rendered unable to respond to or provide possible biochemical apoptosis cues.

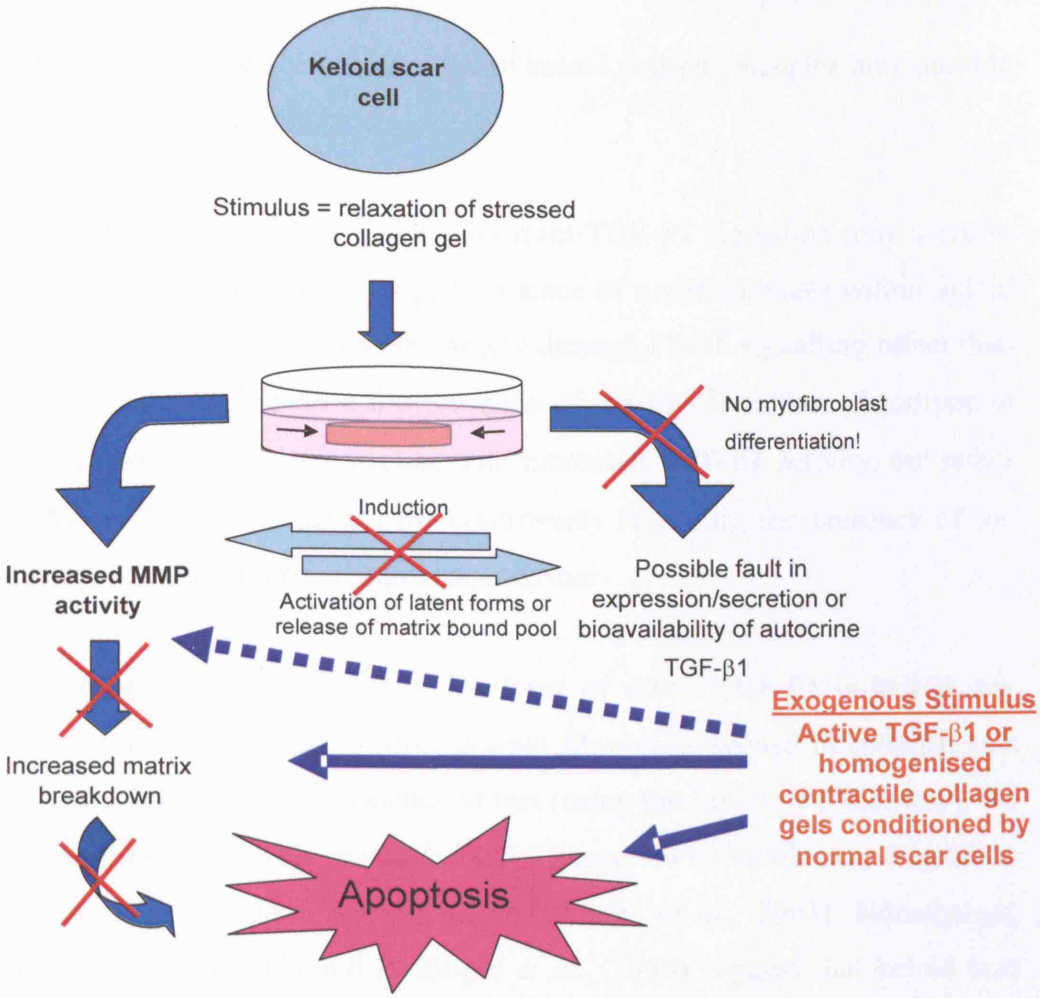


Figure 7.4 Illustrated hypothetical model of the events that take place during collagen contraction by keloid scar fibroblasts. Briefly, keloid scar cells fail to respond to collagen contraction-induced apoptosis, potentially through a failure to remodel the collagen gel and produce matrix breakdown products. However, the addition of exogenous TGF-β1 bypasses this fault, which together with the failure of these cells to differentiate into myofibroblasts during stressed collagen gel culture suggests a failure of autocrine TGF-β1 action either through reduced expression/secretion, activation or increased sequestration into the matrix.

Whether TGF-β1's action are direct or via induction of other factors by the cells also needs to be examined. Future experiments could include assessing the activity of PDGF and CTGF during collagen gel contraction, methods may include: functional studies with siRNA techniques to silence the PDGF and CTGF genes could be used to determine whether they are involved in collagen contraction-induced apoptosis of normal scar cells. In addition, immunoblotting for PDGF and CTGF and other down-stream TGF-β1-activated proteins such as ERK, JNK and p38 MAP Kinase could be carried out with normal scar cell-conditioned contractile collagen gel lysates or the

medium from the gel. Further comparisons with keloid scar cell samples may provide additional clues to keloid pathology.

The hypothesis of keloid scar cells having aberrant TGF-β1 signalling may seem to contradict the observation of the prolonged presence of myofibroblasts within keloid scars. However this characteristic may be largely through PDGF signalling rather than TGF-β1 (Bonner, 2004). Therefore the presence of the myofibroblast phenotype in keloid scars does not necessarily correlate with increased TGF-β1 activity, but rather PDGF availability. This may explain the controversy regarding the presence of (α-SMA expressing) myofibroblasts in keloid scar tissue.

An investigation of the autocrine expression level of active TGF-β1 in keloid scar fibroblasts versus normal scar and normal dermal fibroblasts seeded in collagen gels should be assessed for conclusive evidence of this (using the Luciferase Bioassay). No research has examined the levels of active TGF-β1 secreted by keloid scar fibroblasts alone, only plasma levels have been published (Bayat *et al.*, 2003). Nonetheless, research by Babu *et al.* (1992) and Bettinger *et al.* (1996) suggest that keloid scar fibroblasts exhibit a hypersensitive response to TGF-β1 with regard to their biosynthetic activity and DNA synthesis. This hypersensitivity to TGF-β1 possibly indicates a fault with the secretion or activation of autocrine TGF-β1; keloid scar cells may therefore be hypersensitive to paracrine levels to compensate for this fault. Indeed, any increase in TGF-β1 receptor levels would indicate this. In order to determine if there is a fault in the transcription of the gene or activation of TGF-β1, further experiments could involve: studying the gene expression levels present within keloid scar versus normal scar cell-conditioned contractile collagen gel samples by quantitative RT-PCR, examining the levels of latent- versus active-TGF-β1 present within cell-conditioned contractile collagen gels seeded with keloid scar cells versus normal scar cells by *in situ* immunohistochemistry or western blotting. If keloid scar cell-conditioned contractile collagen gel samples show equivalent TGF-β1 transcription levels and translation into the latent form, the latter experiment would demonstrate whether levels of the activated TGF-β1 protein are comparatively sufficient in keloid scar cell samples compared to that detected with normal scar cell samples.

7.8.1 Supportive Evidence for the Role of TGF-β1 as a Co-Factor to Cells Response to Collagen Contraction

TGF-β1 may affect fibroblasts in the absence of serum or activation factors, in such a way as to prime cells in order for them to respond to biochemical apoptosis cues produced on collagen gel contraction.

The first research groups to discover that apoptosis occurred in contractile collagen gels maintained the gels in 10% serum and proposed that serum factors were required for apoptosis induction (Fluck *et al.*, 1998). Recently, Kobayashi *et al.* (2005) on the other hand, found that serum factors reduced apoptosis.

It was hypothesised at the start of the work carried out in this thesis that in fact serum may contain survival factors that act to inhibit or mask the apoptosis induced during collagen contraction (Chapter 3). Experiments throughout this thesis have involved using minimal serum containing media (1% NGM). In the recent paper by Kobayashi *et al.* (2005), they found that when comparing the degree of apoptosis induced in serum containing media to that in non-serum containing media, the latter showed increased apoptosis. In addition, the degree of apoptosis in contractile gels was further significantly increased with the addition of TGF-β1 to the SFM, which was then significantly decreased with the addition of serum. Potentially, if the work carried out in this thesis had been carried out in serum free media, the apoptosis detected during collagen gel contraction maybe further increased. However, 1% NGM was chosen as it was found to maintain cells relatively quiescent and healthy in anchored gels.

Interestingly, research by Alanko and Saksela (2000) on a different cell type (normal melanocytes) also found that when TGF-β1 was added to cells grown in type-1 collagen gels, apoptosis was dramatically accelerated. This study involved comparing the degree of apoptosis induced in contractile gels in the presence of 10% serum alone or with the addition of TGF-β1 to the 10% serum. In this thesis, contractile collagen gels maintained in 1% serum and 1% + TGF-β1 showed equivalent cell death. It is possible that TGF-β1's role specifically during collagen contraction is to provide pro-apoptotic signals that out compete any anti-apoptotic signals present. As the experiments in this thesis were performed in medium containing minimal survival

factors, it is possible that maximum apoptosis was already taking place in contractile collagen gels, which exogenous TGF-β1 could not exceed. The fact that Kobayashi *et al.* (2005) found increased apoptosis in contractile collagen gels with TGF-β1 in SFM may be due to differences in cell type. What is most intriguing about the results presented in this thesis was the fact that the addition of exogenous TGF-β1 to cells maintained in anchored collagen gels also induced apoptosis. This is in contrast to the findings of Kobayashi *et al.* (2005) who found TGF-β1 had no effect on apoptosis in anchored collagen gels inhabited by fetal lung fibroblasts. Again, this may be due to differences in sensitivity of cells derived from different origins to such conditions.

Kobayashi *et al.* (2005) went on to demonstrate by western blot analysis that the presence of serum was associated with an increase of cIAP-1 and Bcl-2, anti-apoptotic proteins (see section 1.7 for more information on these apoptosis proteins). Interestingly, serum was also associated with an apparent increase in Bax, a pro-apoptotic protein. They suggested therefore, that factors present in serum might be able to affect the balance between pro- and anti-apoptotic factors. Kobayashi *et al.* (2005) also found that TGF-β1 activated caspase-3 and subsequently PARP in contractile collagen gels. It was therefore suggested that TGF-β1 may affect apoptosis through controlling the activity of caspases.

Indeed, results presented in this thesis demonstrate that caspase-3 is cleaved into its active form during collagen contraction. In addition, inhibiting the activity of caspase-3 specifically inhibits the apoptosis induced by collagen contraction and TGF-β1. Kobayashi *et al.* (2005) proposed that that the release of cytochrome c from the mitochondria may be involved in this caspase activation. As described previously in section 1.7, controlling cytochrome c regulation are members of the Bax/Bcl family. In this context, Kobayashi *et al.* (2005) found that TGF-β1 induction of apoptosis was associated with augmented expression of Bax, a pro-apoptotic member of the Bax/Bcl-2 family together with inhibition of Bcl-2, an anti-apoptotic member. Also, TGF-β1 was associated with inhibition of both cIAP-1 and XIAP, two inhibitors of the caspase-cascade.

Research carried out by Derderian *et al.* (2005) found that mechanically-stressed fibroblast-populated collagen gels are also subject to a down-regulation in the expression of the pro-apoptotic gene Bax, down-regulating normal cellular apoptosis.

These results go some way to detailing a possible cellular response to collagen contraction and TGF-β1. Specifically, TGF-β1's role maybe to increase the expression of pro-apoptotic proteins within the cell, in such a manner as to increase the potential for cells to undergo apoptosis.

As previously mentioned, Kobayashi *et al.* (2005), also found that factors inducive to myofibroblast induction other than TGF-β1, such as PDGF, could induce collagen contraction but not apoptosis. This result also demonstrates that the specific effect of TGF-β1 on cells within collagen matrices is specific to TGF-β1 alone, not simply any growth factor capable of inducing myofibroblasts differentiation.

7.9 Potential Distinct Role of TGF-β1 in Keloid Scar Cell Apoptotic Response

It is possible that TGF-β1 is not required as a distinct co-factor for normal scar fibroblasts or dermal fibroblasts to respond to collagen contraction; that it is only needed in this manner by keloid scar cells. TGF-β1 may be acting in a totally different way. The only role TGF-β1 may have in collagen contraction-induced apoptosis of normal scar cells is to induce an up-regulation of MMP activity that mimics that induced during collagen contraction. Results from keloid scar cells demonstrate that TGF-β1 enables the cells to respond to the apoptosis-inducing properties of matrix-breakdown products.

There is a large body of evidence in the literature detailing the 'transforming' nature of TGF-β1, some of which describe that a variety of cells treated with TGF-β1 show certain attributes of transformation, such as escape from the control of density/contact inhibition, anchorage independent growth. Whether this ability of TGF-β1 is involved in the apparent 'de-transformation' of keloid scar cells is unclear. A recent study by Wisdom *et al.* (2005) using 3T3 cells transformed by Ras, Raf and Fos found that treatment with TGF-β1 reverses the changes in gene expression brought about by transformation. Furthermore these changes are essentially confined to genes encoding

components of the extracellular matrix and the cytoskeleton. Indeed, there is evidence for over-expression of c-Fos in both keloid and hypertrophic scars (Teofoli *et al.*, 1999; Hu *et al.*, 2002). In addition, Ras is important for stress fibre assembly, organisation of α-SMA and the myofibroblast morphology (section 5.1.1.3), which are clearly aberrant in keloid scar cells when cultured within collagen matrices (Figure 5.8).

Further investigation could involve assessing the oncogenes: Ras, Raf and Fos in keloid scar cell-conditioned contractile collagen gel samples. Experiments could involve: examination of gene expression levels by quantitative RT-PCR, assessing these genes at the protein level by western blotting, comparing the results with that expressed by normal scar cell samples. Furthermore, the ability of keloid scar fibroblasts to undergo apoptosis to collagen gel contraction could be assessed by siRNA gene silencing techniques or transforming the cells with any oncogenes implicated from the latter experiments. These experiments would provide clues as to whether these oncogenes are involved in the keloid scar pathology.

Finally, since the origin of cells present within a wound is still undetermined, cells derived from normal scar may originate from quiescent dermal fibroblasts or pericytes say, whereas keloid scar cells may originate from a completely different source such as pre-adipocytes or circulating progenitor stem cells. In this way, the cells derived from these keloid scars may be inherently different to that of dermal fibroblasts and normal scar fibroblasts and thus may exhibit different requirements for the induction of their apoptosis. At present there is no known specific cell marker to differentiate between a fibroblast, pericyte or progenitor stem cell and as such this area of research is controversial.

7.10 Concluding Remarks

It is clear from the results presented in this thesis that MMP action is intrinsically linked with the induction of apoptosis in contractile collagen gels, however whether this is strictly through their enzymatic breakdown of matrix components remains to be proven.

This is the first time TGF-β1 has been implicated in the induction of skin fibroblast apoptosis. Considering TGF-β1 is present throughout wound healing this suggests it plays a dual role. Whether, for normal scars, this is via its effect on collagenase activity alone or as a permissive co-factor requires further experimentation. Importantly, TGF-β1 has been previously found to signal human lung fibroblasts to undergo apoptosis in contractile collagen gels (Kobayashi *et al.*, 2005), however it is widely appreciated that cells derived from different origins behave very differently.

Finally, keloid scar cells were shown to exhibit a distinct defect in their ability to respond to the specific apoptosis cues produced during collagen contraction. Of even more relevance to the keloid pathology, is the finding that active TGF-β1 is required to enable these cells to undergo apoptosis in collagen gels. What is clear is that anti-scarring approaches that concentrate on simply blocking the action of TGF-β1 may in some cases do more harm than good. This may be particularly true for pathological scarring.

Appendices

APPENDIX I

List of Antibodies, Growth Factors and Inhibitors Used

Reagent	Company	Concentration/ Dilution factor
Mouse monoclonal GAPDH	Abcam	1:5000
Monoclonal anti-human TGF-β1 antibody	R & D	2ng/ml
Mouse monoclonal α-SMA antibody	Sigma, clone 1A4	1:1000
Mouse monoclonal caspase-3 antibody	Cell Signalling	1:1000
Mouse monoclonal anti-PARP-1	Calbiochem, Ab-2	1:1000
Mouse monoclonal P53	Dako, D07clone	1:100
Rabbit anti-mouse biotinylated antibody	Dako	1:1000
Streptavadin Alkaline Phosphatase	Dako	1:1000
Recombinant human TGF-β1	R & D	2ng/ml
Recombinant human PDGF	R & D	20ng/ml
Recombinant human TIMP-2	Calbiochem	2nM
PMSF (0.1M)	Fluka Biochemika	1mM
Aprotinin	Ferring Pharmaceuticals	100IU
Mannose-6-phosphate (100mM)	Sigma	100μM
Ilomastat (GM6001)	Chemicon International	5μM
MMP-2 specific inhibitor	Chemicon International	10μM
Caspase-3 Inhibitor I	Calbiochem	5nM

APPENDIX II

GENERAL PRECAUTIONS USED FOR RNA EXTRACTION

A separate clean "RNA area" lab bench was designated for RNA extraction. This was cleaned with IMS, and then cleaned further with DEPC water (see below). A clean laboratory coat and fresh gloves were utilised on a regular basis. All equipment and apparatus was designated for RNA use only and was pre-incubated with DEPC-water before autoclaving. (DEPC water is ultrapure water i.e. DNAase and RNAase free).

REAGENTS FOR RNA EXTRACTION

DEPC WATER

- 2ml diethylprocarbonate (0.2%) was added to 1 litre ultrapure (18MΩ) water shaking vigorously
- This was incubated overnight at 37°C
- Autoclaved
- Then stored at room temperature until use

GUANIDIUM THIOCYANATE (GT) EXTRACTION BUFFER
(Store at 4°C for 2 months)

FORMULATION	SOURCE	For 500ml
4M guanidium thiocyanate (GT)	Sigma	236.4g
0.5% sarkosyl (free acid)	Sigma	2.5g
0.1% antifoam A	Sigma	0.5ml
25mM sodium citrate pH 7.0	BDH	50ml of 250mM stock
0.1M β-mercaptoethanol	BDH	3.49ml
0.2% DEPC	Sigma	1ml

- Dissolved GT in 200ml of DEPC-water at 60°C, stirring thoroughly for 30 minutes
- Separately dissolved Sarkosyl in the sodium citrate stirring thoroughly at room temperature, for 30 minutes
- Mixed the sodium citrate-Sarkosyl plus the GT solution in the fume cupboard
- Then added the antifoam A
- The solution was made up to 500ml with dH_2O using a measuring cylinder
- The pH was adjusted to 7.0
- 0.2% DEPC was then added to the solution, shaking vigorously to dissolve
- This solution was incubated at 37°C overnight
- Autoclaved
- Finally β-mercaptoethanol was added

WATER-SATURATED PHENOL

- 100g of molecular grade phenol (Sigma D5758) was melted at 60°C, then allowed to cool
- The bottle was filled to the top with an equal volume of DEPC water and shaken vigorously
- The phenol was left overnight to allow phase separation
- The aqueous phase was removed and stored in the dark (foil wrapped bottle) at 4°C

ISOAMYLALCOHOL

- Isoamylalcohol (BDH 100383L)
- Chloroform (BDH 100774W)
- Mixed chloroform with isoamylalcohol at a ratio of 24:1 in a clean autoclaved container

SODIUM ACETATE

- 2M sodium acetate was adjusted to pH 4 with acetic acid.
- 12.31g sodium acetate and
- 48.75ml of acetic acid was added to the sodium acetate solution
- Made up to 500ml with DEPC water

SODIUM CITRATE

- 36.76g of tri-sodium citrate was made up to 500ml in DEPC water, and adjusted to pH 7.0 with acetic acid

TAE

1 litre stock solution of 50x TAE was made as follows

- 242g Tris base (Sigma)
- 57.1ml glacial acetic acid
- 18.5g EDTA (ethylenediaminetetacetic acid) (Sigma)
- 20g sodium acetate
- 700mls of distilled water was added to the above ingredients in a conical flask swirling gently
- The solution was then adjusted to pH 8

APPENDIX III

Reagents for Reverse Transcriptase Reaction

Reagent	Quantity	Volume	Source
Extracted RNA	5μg	8μl	-
Oligo-DT primers	200μg/ml	1μl	Gibco
0.1M DTT		2μl	Gibco
5 x RT buffer		4μl	Gibco
DEPC water		1μl	-
10mM dNTPs		2μl	Pharmacia (100mM kit)
RNA guard	30U/μl	1μl	Pharmacia (porcine)
MMLV Reverse transcriptase (RT)	200U/ml	1μl	Gibco
TOTAL VOLUME		**20 μl**	-

Table showing reaction substrates for the RT reaction for cDNA synthesis

Number of cycles	Temperature °C	Duration / minutes
1	65	10
1	0	5
1	37	60
1	75	10

Table demonstrating reverse transcriptase reaction conditions

Reagents for the Polymerase Chain Reaction

Reagent	Quantity	Source
cDNA (from RT reaction)	2µl	-
10 x PCR buffer	2µl	Finnzymes
2mM dNTP	2µl	Invitrogen
DEPC water	8.75µl	-
DMSO	1µl	Sigma
Primer1 (For)	2µl (10pmol)	Applied Biosystems
Primer 2 (Rev)	2µl (10pmol)	Applied Biosystems
GAPDH primer (For)	2µl (10pmol)	Applied Biosystems
GAPDH primer (Rev)	2µl (10pmol)	Applied Biosystems
DNAzyme	0.25µl	Invitrogen
TOTAL VOLUME	**20µl**	

Table showing reaction substrates for the polymerase chain reaction

PCR CONDITIONS

Number of cycles	Temperature °C	Duration / minutes
30	95	1
	Annealing temp -2°	2
	72	3
1	72	7

Table demonstrating PCR reaction conditions

APPENDIX IV

Table showing primer sequences for target genes for PCR, their expected PCR product size, and Tm annealing temperatures for each gene investigated.

PRIMER	SEQUENCE	PCR PRODUCT SIZE (BP)	Tm
MMP-1	F-5' CGACTCTAGAAACACAAGAGC 3'	781	62
	R–5' GGTTAGCTTACTGTCACACGC 3'		64
MMP-2	F-5' GTGCTGAAGGACACACTAAAG 3'	598	62
	R-5' CCATCCTTCTCAAAGTTGTAG 3'		64
MMP-3	F-5' GAACAATGGACAAAGGATACAACA 3'	732	60
	R-5' TTCTTCAAAAACAGCATCAATCTT 3'		62
MMP-13	F-5' TGCTGGCTCATGCTTTTCCTC 3'	273	64
	R-5' GGTTGGGGTCTTCATCTCCTG 3'		66
TIMP-1	F-5' ACCTTATACCAGCGTTATGAG 3'	356	60
	R-5' AGCTGGTCCGTCCACAAGCA 3'		64
TIMP-2	F-5' CGCTGGACGTTGGAGGAAA 3'	349	60
	F-5' GTCCTCGATGTCGAGAAACT 3'		60
TIMP-3	F-5' GCCTTCTGCAACTCCGAC 3'	453	58
	R-5' GTAGTGTTTGGACTGGTAG 3'		56
TIMP-4	F-5' CTGCTGACACTGAAAAAATG 3'	485	56
	R-5' CTAGGGCTGAACGATGTC 3'		56
GAPDH	F-5' CTCTGGTAAAGTGGATATTG 3'	216	56
	R-5' AGTGGACTCCACGACGTACT 3'		62

N.B. All sequences checked on BLAST, and alignment checked. All optimised for 60°C PCR cycle.

The annealing temperature is dependant on the length and composition of the primers and ideally should be between 1 and 5°C lower than the lowest Tm value (Warford *et al.*, 1988). Too low a Tm will result in non-specific annealing and therefore non-specific amplification; too high a Tm leads to reduced yield.

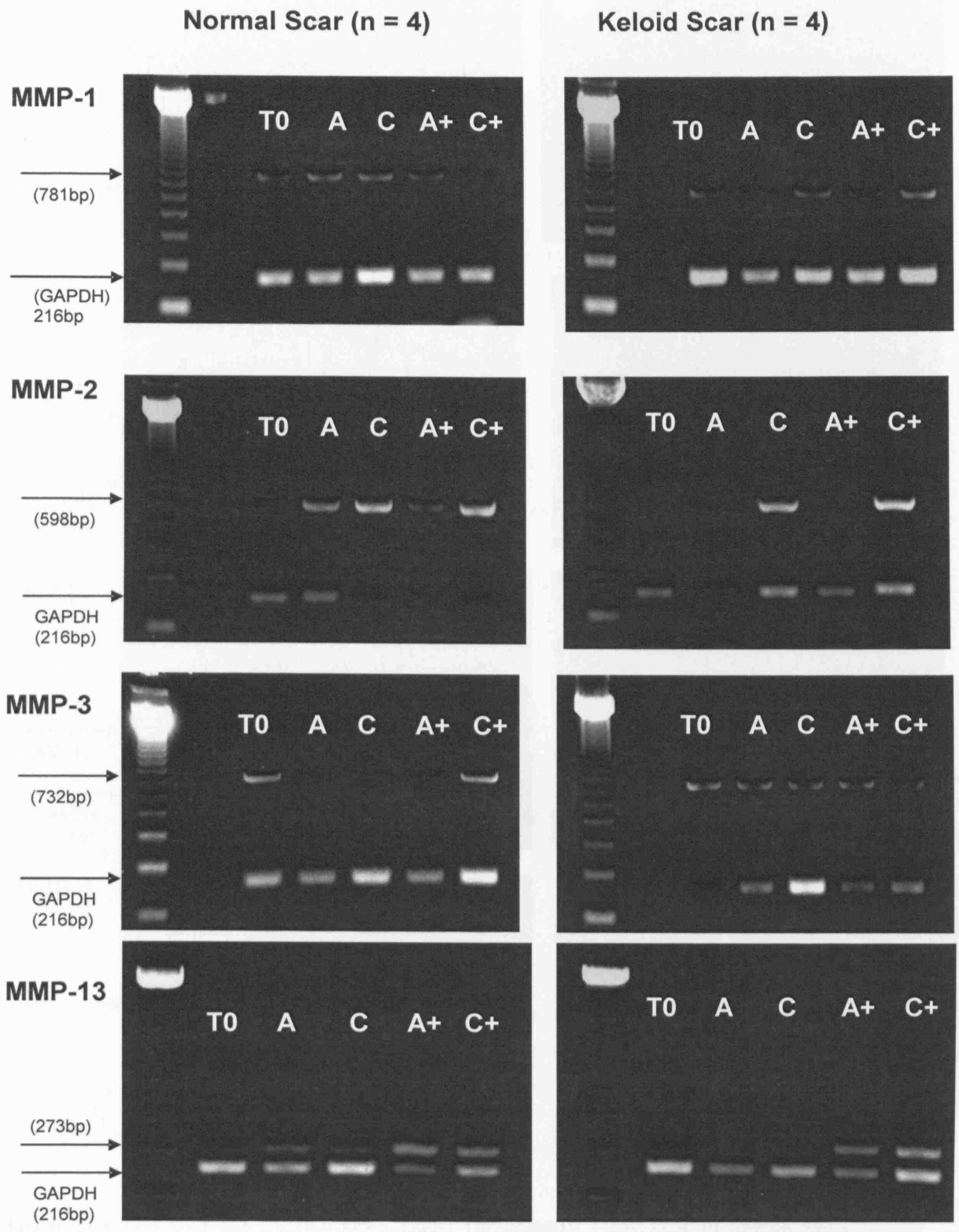
Primer Tm was calculated by the equation

$$Tm(°C) = 4(G+C) + 2(A+T)$$

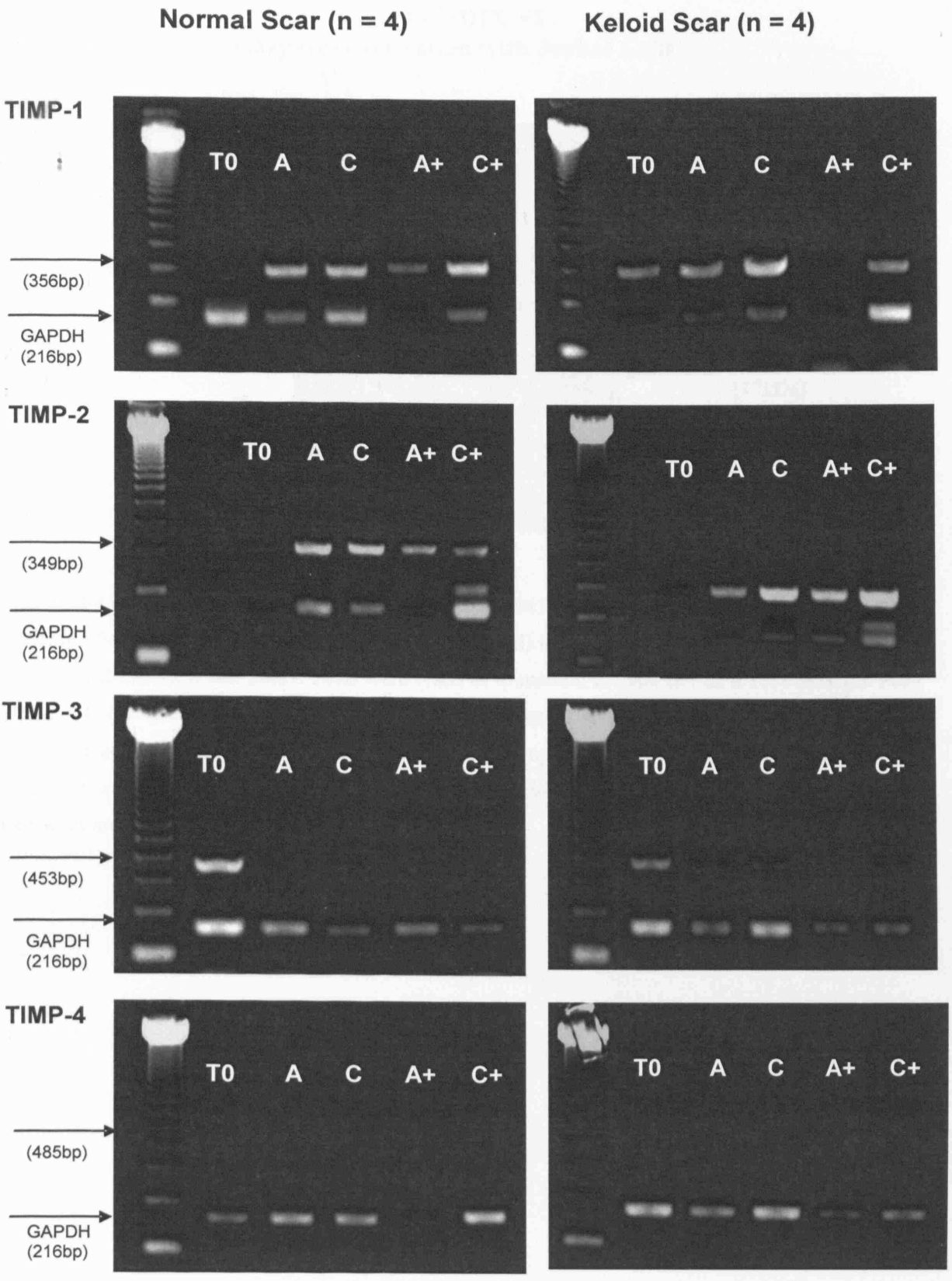
Where G, C, A and T are the number of the equivalent nucleotides found in the primer.

APPENDIX V

Typical PCR gels

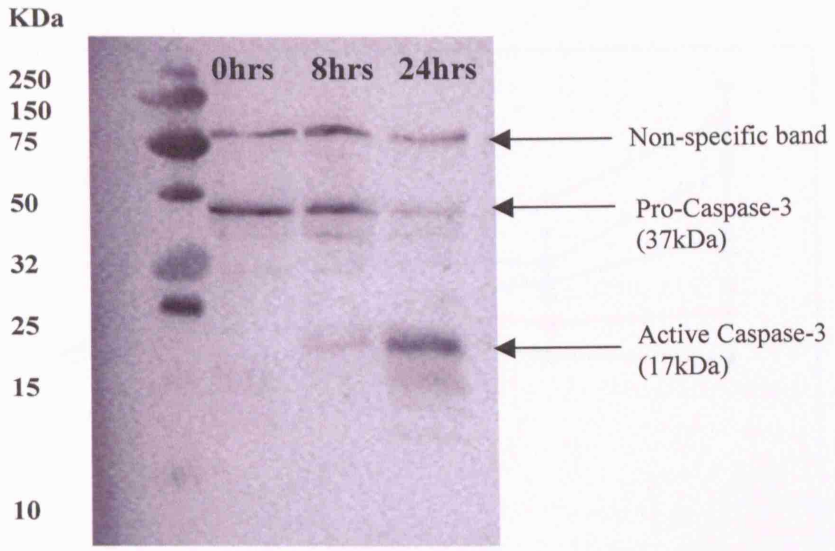


Typical PCR gels showing 1,2,3Kb DNA ladder (Invitrogen), MMP gene of interest and GAPDH. Samples analysed included: T0, anchored (A) and contractile (C) day-7 collagen gels that had been treated with TGF-β1 (+) or without.



Typical PCR gels showing 1,2,3Kb DNA ladder (Invitrogen), TIMP gene of interest and GAPDH. Samples analysed included: T0, anchored (A) and contractile (C) day-7 collagen gels that had been treated with TGF-β1 (+) or without.

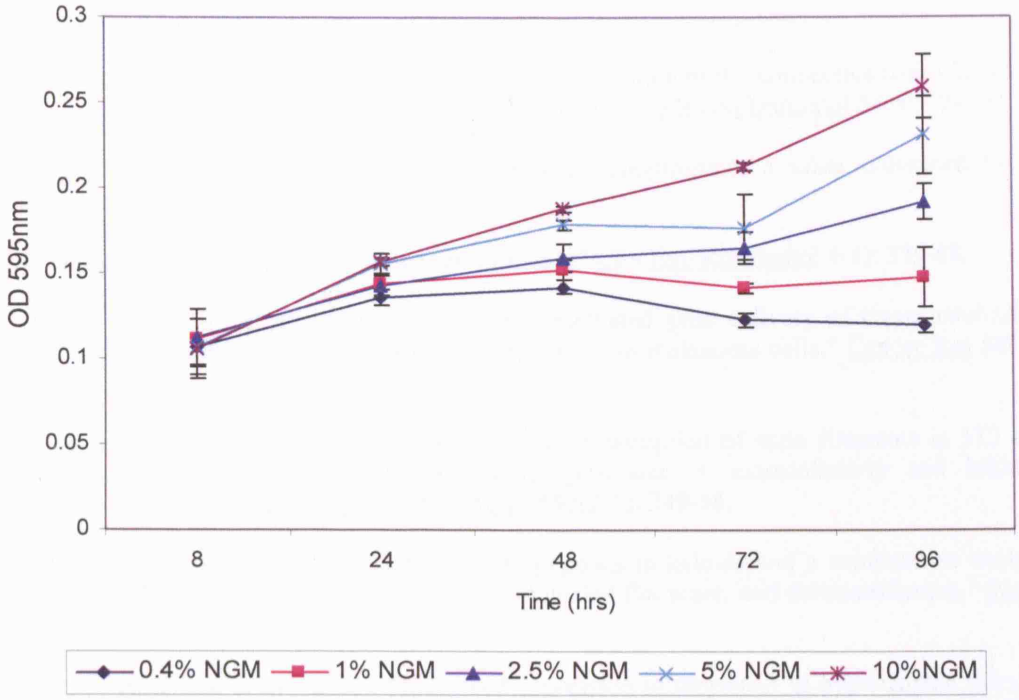
APPENDIX VI
Caspase-3 activation with Jurkat Cells



Western blot analysis was carried out with Jurkat cells as in method 2.7 to demonstrate clear caspase-3 activation after incubating cells with Doxorubicin (1mg/ml) in serum free media. Cells were incubated with Doxorubicin for 8 and 24hrs. Cells were lysed as in method 2.7 and run on a 15% SDS gel. Pro-caspase-3 (37kDa) and active caspase-3 (17kDa) was detected with a mouse monoclonal caspase-3, using three-layer antibody detection method (detailed in appendix I). At 24hrs strong activation of caspase-3 was detected, concomitant with a reduction in pro-caspase-3. This experiment was repeated three times and the above is a representative gel.

APPENDIX VII

FCS titration assay



Titration of fetal calf serum (FCS) analysing normal scar cell number over a period of 4-days. Crystal Violet staining was used to assess live cell number over time. A concentration of 1% FCS (1% NGM) was found to keep cells quiescent over the time-course compared to the other concentrations of FCS studied.

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